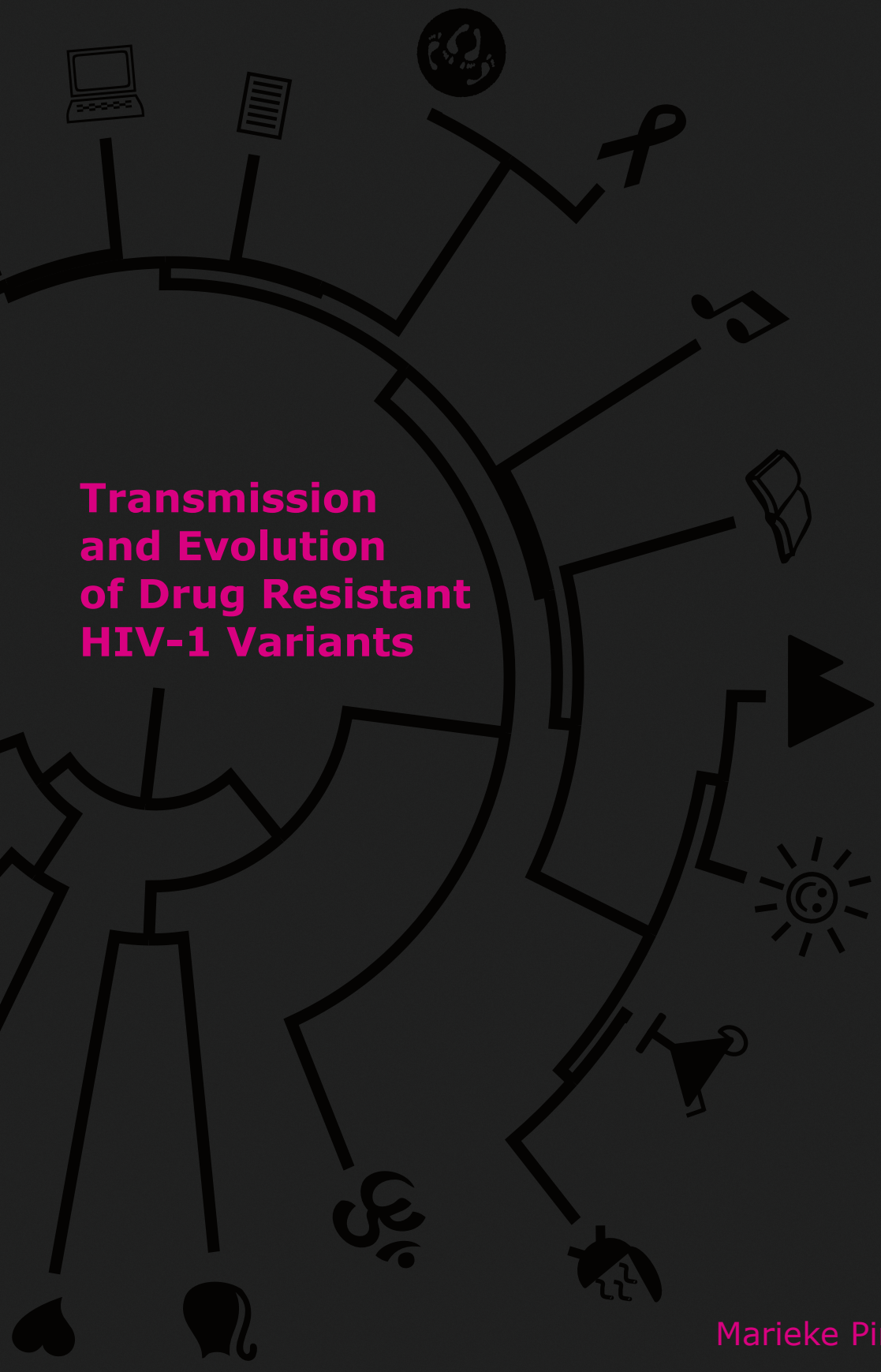


Transmission and Evolution of Drug Resistant HIV-1 Variants

Marieke Pingen

Transmission and Evolution of Drug Resistant HIV-1 Variants

Marieke Pingen

Transmission and Evolution of Drug Resistant HIV-1 Variants

PhD Thesis, Erasmus Medical Center, the Netherlands

Layout and printing: Off Page, Amsterdam, the Netherlands

Cover: Marieke Pingen

Doodle: Caroline M.M. Gits

ISBN: 978-94-6182-252-9

The research described in this thesis was financially supported by the Dutch Aids fonds [grant 2007043] and partially performed at the University Medical Center Utrecht.

Printing of this thesis was financially supported by ViiV HC Nederland, Virology Education, Merck Sharp & Dohme and Boehringer Ingelheim bv.

University Medical Center
Utrecht

Transmission and Evolution of Drug Resistant HIV-1 Variants

Overdracht en evolutie van medicijn resistente HIV-1 varianten

(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de rector magnificus prof.dr. H.G. Schmidt en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op 8 mei 2013 om 11.30 uur

door

Marieke Pingen
geboren op 22 september 1984 te Nijmegen

Promotor:	Prof. dr. C.A.B. Boucher
Co-promotoren:	Dr. A.M.J. Wensing Dr. M. Nijhuis
Commissie:	Prof. dr. A.D.M.E. Osterhaus Prof. dr. E.J. Wiertz Prof. dr. A. Verbon
	Prof. dr. D.D. Richman Prof. dr. H.A. Verbrugh Prof. dr. E.C.M. van Gorp Dr. G.M.G.M. Verjans

Index

Chapter 1

General Introduction

Transmission of HIV

At the end of 2010, an estimated 34 million people were living with human immunodeficiency virus type 1 (HIV-1, referred to as HIV), the causative agent of acquired immunodeficiency syndrome (AIDS). Although the worldwide rate of new infections is declining, the number of people living with HIV is still increasing. In Western and Central Europe and in North America, the incidence of HIV remains constant resulting in an estimated 2.2 million people living with HIV in 2010. However, the number of aids-related deaths has been stable for the past ten years in these areas. This is largely due to increasing access to suppressive HIV treatment[1].

Although HIV can be transmitted vertically from mother to child or via contaminated blood products and needles, the vast majority of individuals are infected via sexual contact. The rate of HIV transmission per coital act is influenced by many factors such as stage of the infection of the index patient, plasma HIV RNA, type of sexual contact and co-infections in both index patient and recipient. The probability of HIV transmission per sexual event is low[2]. Although only one or very few virions initiate the infection of the new host[3, 4], shortly after establishment of the infection the viral population rapidly expands to a very large population[5]. As the error rate of HIV reverse transcriptase during HIV replication is high[6] and recombination events are frequent[7], the HIV population becomes a heterogenic and dynamic viral population consisting of genetically distinct, but closely related HIV variants: the quasispecies. Within the quasispecies, wild type is defined as the most fit and most common variant.

Antiretroviral Therapy

Before 1996, only a limited number of drugs were available to treat HIV and therapy consisted of one or two nucleoside reverse transcriptase inhibitors (NRTIs). These therapy regimens often caused selected of mutations conferring drug-resistance[8, 9]. After the approval of the first protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitor (NNRTI) in 1996, highly active antiretroviral therapy (HAART) became available. The combination of three drugs from at least two drug classes not only resulted in a rapid decrease of HIV replication and increase of $CD4^+$ T cell counts, it also reduced the mortality and morbidity related to HIV infection and AIDS[10-12]. However, initial HAART also led to the selection of multi-drug resistant HIV variants[13]. Currently, optimization of HAART regimens has resulted in successful viral suppression with a low pill burden and limited side effects in the vast majority of patients. In addition, increased viral suppression led to a decreased selection of multi-drug resistant HIV variants[14, 15].

Transmission of Drug-Resistant HIV variants

Unfortunately drug-resistant HIV variants can also be transmitted. Several years after the approval of AZT for HIV treatment, the first case of drug-resistant HIV transmission was reported[16]. Epidemiological studies in cities in the United States investigating newly infected patients in the late nineties revealed increasing transmission rates of drug-resistant HIV variants[17, 18]. In the same period in Europe, the retrospective CATCH study showed that 1 in 10 newly diagnosed patients were diagnosed with HIV variants harbouring drug-resistance mutations[19]. More recently, the large, prospective European study SPREAD demonstrated that the prevalence of viruses harbouring transmitted drug-resistance mutations remains stable just below 10% between 2002 and 2007[20].

As transmission of drug resistant HIV variants is common and can contribute to therapy failure[21], current guidelines recommend performing a resistance test of protease and reverse transcriptase on the earliest available sample[22]. Resistance tests in clinical practise and epidemiological studies are usually conducted by population sequencing which can detect viral minorities making up 10-20% of the quasispecies. More sensitive assays can detect viral variants harbouring resistance mutations present in less than 1% of the viral population. The use of more sensitive methods leads to an increased detection of transmitted drug resistant HIV variants[23].

Scope of this Thesis

The aim of this thesis was to investigate the transmission and evolution of drug resistant HIV variants. NRTIs, NNRTIs and PIs were not only the first available drug classes, but are still most commonly used in clinical practise. Therefore, we focussed our studies on drug resistance in protease and reverse transcriptase.

In **Chapter 2**, we investigated the impact of the replicative capacity on the transmission efficacy of drug resistant HIV variants. Next, we have explored the evolution of drug-resistant variants after transmission. An extensive literature search was performed in **Chapter 3** and evolution was investigated in more detail in **Chapter 4**. Furthermore, we have studied the clinical impact of transmission of drug-resistant variants. A transmission cluster containing eight patients diagnosed with an HIV variant harboring several drug resistance mutations was studied in detail in **Chapter 5**. In **Chapter 6**, we investigated the viral quasispecies in a group of patients diagnosed with HIV variants with a single drug resistance mutation. In **Chapter 7** we explored the impact of M41L, a drug resistance mutation frequently observed in transmitted HIV variants. In **Chapter 8**, a case of superinfection is researched.

References

1. UNAIDS. How to get to zero: Faster. Smarter. Better. http://www.unaids.org/en/media/unaids/contentassets/documents/unaidspublication/2011/JC2216_WorldAIDSday_report_2011_en.pdf (August 21st, 2012.
2. Wawer MJ, Gray RH, Sewankambo NK et al. Rates of HIV transmission per coital act, by stage of HIV infection, in Rakai, Uganda. *J Infect Dis* 2005; **191**: 1403-9.
3. Miller CJ, Li Q, Abel K et al. Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. *J Virol* 2005; **79**: 9217-27.
4. Keele BF, Giorgi EE, Salazar-Gonzalez JF et al. Identification and characterization of transmitted and early founder virus envelopes in primary HIV infection. *Proc Natl Acad Sci U S A* 2008; **105**: 7552-7.
5. Little SJ, McLean AR, Spina CA et al. Viral dynamics of acute HIV infection. *J Exp Med* 1999; **190**: 841-50.
6. Mansky LM, Temin HM. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *J Virol* 1995; **69**: 5087-94.
7. Onafuwa-Nuga A, Telesnitsky A. The remarkable frequency of human immunodeficiency virus type 1 genetic recombination. *Microbiol Mol Biol Rev* 2009; **73**: 451-80, Table of Contents.
8. Boucher CA, Tersmette M, Lange JM et al. Zidovudine sensitivity of human immunodeficiency viruses from high-risk, symptom-free individuals during therapy. *Lancet* 1990; **336**: 585-90.
9. Larder BA, Kemp SD. Multiple mutations in HIV reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* 1989; **246**: 1155-8.
10. D'Aquila RT, Hughes MD, Johnson VA et al. Nevirapine, zidovudine, and didanosine compared with zidovudine and didanosine in patients with HIV infection. A randomized, double-blind, placebo-controlled trial. National Institute of Allergy and Infectious Diseases AIDS Clinical Trials Group Protocol 241 Investigators. *Ann Intern Med* 1996; **124**: 1019-30.
11. Collier AC, Coombs RW, Schoenfeld DA et al. Treatment of human immunodeficiency virus infection with saquinavir, zidovudine, and zalcitabine. AIDS Clinical Trials Group. *N Engl J Med* 1996; **334**: 1011-7.
12. Palella FJ, Jr., Delaney KM, Moorman AC et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med* 1998; **338**: 853-60.
13. Richman DD, Morton SC, Wrin T et al. The prevalence of antiretroviral drug resistance in the United States. *AIDS* 2004; **18**: 1393-401.
14. Gill VS, Lima VD, Zhang W et al. Improved virological outcomes in British Columbia concomitant with decreasing incidence of HIV type 1 drug resistance detection. *Clin Infect Dis* 2010; **50**: 98-105.
15. Vercauteren J, Deforche K, Theys K et al. The incidence of multidrug and full class resistance in HIV infected patients is decreasing over time (2001-2006) in Portugal. *Retrovirology* 2008; **5**: 12.
16. Erice A, Mayers DL, Strike DG et al. Brief report: primary infection with zidovudine-resistant human immunodeficiency virus type 1. *N Engl J Med* 1993; **328**: 1163-5.
17. Grant RM, Hecht FM, Warmerdam M et al. Time trends in primary HIV drug resistance among recently infected persons. *JAMA* 2002; **288**: 181-8.
18. Little SJ, Holte S, Routy JP et al. Antiretroviral-drug resistance among patients recently infected with HIV. *N Engl J Med* 2002; **347**: 385-94.

19. Wensing AM, van de Vijver DA, Angarano G et al. Prevalence of drug-resistant HIV variants in untreated individuals in Europe: implications for clinical management. *J Infect Dis* 2005; **192**: 958-66.
20. Programme DFobotS. Recent dynamics of transmitted drug resistance in Europe: SPREAD programme 2006-2007. *9th European Workshop on HIV & Hepatitis Treatment Strategies & Antiviral Drug Resistance*. Paphos, Cyprus: Reviews in Antiviral Therapy & Infectious Diseases, 2011; 15-6.
21. Wittkop L, Gunthard HF, de Wolf F et al. Effect of transmitted drug resistance on virological and immunological response to initial combination antiretroviral therapy for HIV (EuroCoord-CHAIN joint project): a European multicohort study. *Lancet Infect Dis* 2011; **11**: 363-71.
22. Vandamme AM, Camacho RJ, Ceccherini-Silberstein F et al. European recommendations for the clinical use of HIV drug resistance testing: 2011 update. *AIDS Rev* 2011; **13**: 77-108.
23. Wainberg MA, Moisi D, Oliveira M et al. Transmission dynamics of the M184V drug resistance mutation in primary HIV infection. *J Antimicrob Chemother* 2011; **66**: 2346-9.

Chapter 2

Diminished Transmission of Drug Resistant HIV-1 Variants with Reduced Replicative Capacity in a Human Transmission Model

M. Pingen[1,2]*, R. Sarrami Forooshani[3]*, A.M.J. Wensing[1], P.M. van Ham[1], C.A.B. Boucher[2], T.B.H. Geijtenbeek[3] and M. Nijhuis[1]

[1] Virology, Department of Medical Microbiology, UMC Utrecht, Utrecht, the Netherlands
[2] Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands
[3] Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, the Netherlands.

* These authors contributed equally to this work

Submitted

Abstract

Different patterns of drug resistance are observed in treated and therapy naïve HIV-1 infected populations. Especially the NRTI-related M184I/V variants, which are among the most frequently encountered mutations in treated patients, are underrepresented in the antiretroviral naive population. M184I/V mutations are known to have a profound effect on viral replication and tend to revert over time in the new host. However, it is debated whether a diminished transmission efficacy of HIV variants with a reduced replication capacity can also contribute to the observed discrepancy in genotypic patterns.

As dendritic cells (DCs) play an pivotal role in HIV-1 transmission, we used a model containing primary human Langerhans cells (LCs) and DCs to compare the transmission efficacy M184 variants (HIV-M184V/I/T) to HIV wild type (HIV-WT). As control, we used HIV harbouring the NNRTI mutation K103N (HIV-K103N) which has a minor effect on replication and is found at a similar prevalence in treated and untreated patients.

In comparison to HIV-WT, the HIV-M184 variants were less efficiently transmitted to $CCR5^+$ Jurkat T cells by both LCs and DCs. The transmission rate of HIV-K103N was slightly reduced to HIV-WT in LCs and even higher than HIV-WT in DC. Replication experiments in target cells revealed no apparent differences in replicative capacity between the mutant viruses and HIV-WT. However, the infection rate of LCs and DCs was in concordance with the transmission results; infection by HIV-M184 variants was lower than infection by HIV-WT, and the level of infection by HIV-K103N was intermediate for LCs and higher than HIV-WT for DCs.

Our data demonstrates that drug resistant M184-variants display a reduced replicative capacity in LCs and DCs which directly impairs their transmission efficacy. As such, diminished transmission efficacy contributes to the lower prevalence of drug resistant variants in therapy naive patients.

Introduction

HIV variants harbouring drug-resistance mutations are detected in approximately 10% of all newly diagnosed patients in the Western world[1, 2]. Large clinical studies indicated that transmitted drug resistance may impact virological and immunological response to initial antiretroviral therapy[3, 4]. A change from a methionine to valine or isoleucine at position 184 (M184V/I) in reverse transcriptase (RT) is the most frequently observed resistance mutation in patients experiencing treatment failure[5-7] but is only rarely observed in untreated, newly diagnosed individuals using population-based sequencing assays[1, 2, 5]. M184V/I causes high level resistance against the nucleoside reverse transcriptase inhibitors lamivudine and emtricitabine, but at the same time considerably decreases the processivity of reverse transcriptase (RT) resulting in a reduced viral replicative capacity (RC)[8, 9]. Contrary to M184V, the frequently observed RT mutation K103N has a similar prevalence in treated and untreated patients[5]. The presence of K103N causes high levels of resistance against the non-nucleoside reverse transcriptase inhibitors efavirenz and nevirapine. K103N has only a modest effect on viral RC[10, 11,] and can persist for years after transmission to a new host[12].

The low prevalence of the M184V mutation in therapy-naive individuals may be explained by the reduced RC of this mutant, which can directly impair transmission efficacy and/or lead to reversion of M184V in the new host. When HIV variants harbouring M184V are transmitted to a new host, rapid reversion of the M184V variant has been documented (reviewed in 12). Accordingly, the M184V variant can be detected as a minority species in recently infected individuals using very sensitive assays, which is suggestive of reversion to wild type[5]. In addition, diminished transmission efficacy of the M184V variant has been suggested based on mathematical modelling and a macaque SHIV model[13, 14]. However, the impact of RC on transmission efficacy has never been investigated in a human transmission model.

Sexual HIV transmission is an inefficient process during which one or only a limited number of virions initiate an infection in a new host, resulting in a severe transmission bottleneck[15, 16]. Although $CD4^+$ T cells are the predominant target cells of HIV, it has been postulated that dendritic cells (DCs) naturally residing in the genital mucosa play a major role during sexual transmission[17-19]. Within the genital mucosa, Langerhans cells (LCs) reside in the epithelial layer and are the first DC subset encountered by HIV. LCs express the C-type lectin receptor langerin that captures HIV, leading to internalization and degradation of HIV. LCs therefore function as a natural barrier against HIV transmission[20]. However, when the protective function of langerin is saturated, for example in the presence of a high inoculum or when langerin is downregulated due to cell maturation, LCs can become infected and subsequently transmit HIV to T cells.

Furthermore, DC-SIGN$^+$ DCs, which reside in the sub-epithelium, can transmit HIV to T cells. These DCs express the C-type lectin DC-SIGN that efficiently captures HIV and transmits the virus to T cells[21]. Transmission by DCs occurs either as a result of infection of DCs and subsequent *de novo* virus production (in *cis*), or by uptake and transfer of virions (in *trans*)[15].

The objective of this study was to investigate the transmission efficacy of the HIV-1 M184V/I RT variants. We used an HIV transmission model containing primary human DCs to compare the transmission efficacy of HIV harbouring M184V/I to wild type HIV. With this virus panel, we demonstrated that the M184 variants were less efficiently transmitted to CCR5$^+$ Jurkat T cells by both LCs and DCs, which was due to the lower RC of the M184 variants in both DC subsets. These results clearly imply a role for HIV RC in transmission efficacy and provide an additional explanation for the low prevalence of HIV M184V/I in therapy naïve individuals.

Results

Impact of drug resistance mutations on transmission by LCs and DCs

We hypothesized that due to a diminished replication potential[9], HIV harbouring M184V/I is less efficiently transmitted than HIV-WT or drug resistant virus variants with a high RC[11]. Therefore, we compared the transmission efficacy of HIV-M184V/I to HIV-WT and HIV-K103N. To gain more insight in the impact or RC on transmission efficacy, we additionally investigated the drug resistant HIV-M184T variant. The RC of HIV-M184T is even more impaired than the RC of M184V/I, and as such this variant is rarely observed *in vivo* but can be selected *in vitro*[9, 22]. Since DCs play an important role during HIV transmission[17, 18], we assessed the transmission efficacy of this virus panel by two subsets of DCs: primary human LCs and human monocyte-derived immature DCs.

Migratory LCs were isolated from the epidermis of skin obtained after plastic surgery from multiple healthy donors (purity: >95%, described in ref 23) and exposed to HIV for four days to enable infection. To mimic HIV transmission, the exposed LCs were extensively washed and subsequently co-cultured with CCR5$^+$ Jurkat T cells for two days. Infection of target cells was determined by intracellular p24 staining using flow cytometry. HIV-M184V, -I and –T (combined: HIV-M184 variants) were less efficiently transmitted by LCs than the HIV-WT, whereas the level of transmission of HIV-K103N was intermediate (Figure 1A).

Monocyte-derived immature DCs were used as a model for sub-epithelial DCs[24]. Transmission by DCs obtained from multiple donors (purity: >90%, described in ref 20) was investigated in the aforementioned transmission model. In accordance with the results obtained by LCs, transmission of HIV-M184 variants from DCs to

target cells was lower than HIV-WT (Figure 1B) for 3/4 donors. The transmission rate of HIV-K103N was even higher than HIV-WT in all but one experiment.

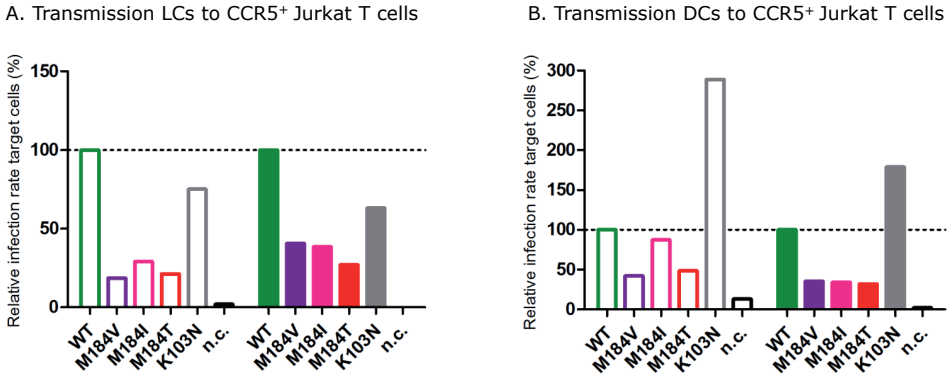


Figure 1. Diminished transmission of M184 variants by LCs and DCs. LCs (A) or DCs (B) were exposed to the equivalent of 17.5 (open bars) or 100 ng (closed bars) p24 for four days, extensively washed and co-cultured for two days with CCR5+ Jurkat T cells. Cells were stained with antibodies against CD1a and intracellular p24, infection was analyzed by flow cytometry. The relative percentage of p24 positive cells compared to the percentage of cells infected by HIV-WT is depicted. Data are representative for 2 (LCs) or 4 (DCs) independent experiments. Abbreviations: WT: HIV-WT, M184V: HIV-M184V, M184I: HIV-M184I, M184T: HIV-M184T, K103N: HIV-K103N, n.c.: no infection control.

Replicative capacity of mutant viruses in target cells

Although the RC of HIV-M184V and HIV-M184I is decreased in primary T cells, we have previously shown that the RC of the mutants is similar to HIV-WT in a T cell line[9]. To confirm that the observed differences in transmission efficacy are caused by transmission from DCs rather than replication in the target cells, we assessed the RC of all viruses in the CCR5+ Jurkat T cells that were used in the transmission experiments. No apparent differences in RC were observed between the mutant and wild type viruses (Figure 2A). As an additional control to minimize the impact of potential differences in replication in target cells, the transmission experiments were repeated using different target cells and a single cycle read out. Therefore, LCs and DCs were exposed to the HIV variants as aforementioned, but replication in TZM-bl target cells was limited to one round of replication by (pre-)incubation with the protease inhibitor indinavir. In line with the results obtained with CCR5+ Jurkat T cells, HIV-M184 variants and HIV-K103N were less efficiently transmitted by LCs than HIV-WT (Figure 2B). The transmission efficacy of DCs to TZM-bl target cells was also comparable to the CCR5+ Jurkat T cells; the HIV-M184 variants were less frequently transmitted as HIV-WT and the HIV-K103N transmission efficacy was comparable to HIV-WT

(Figure 2C). These data suggest that the diminished transmission of M184 variants by LCs and DCs is not caused by replication in target cells.

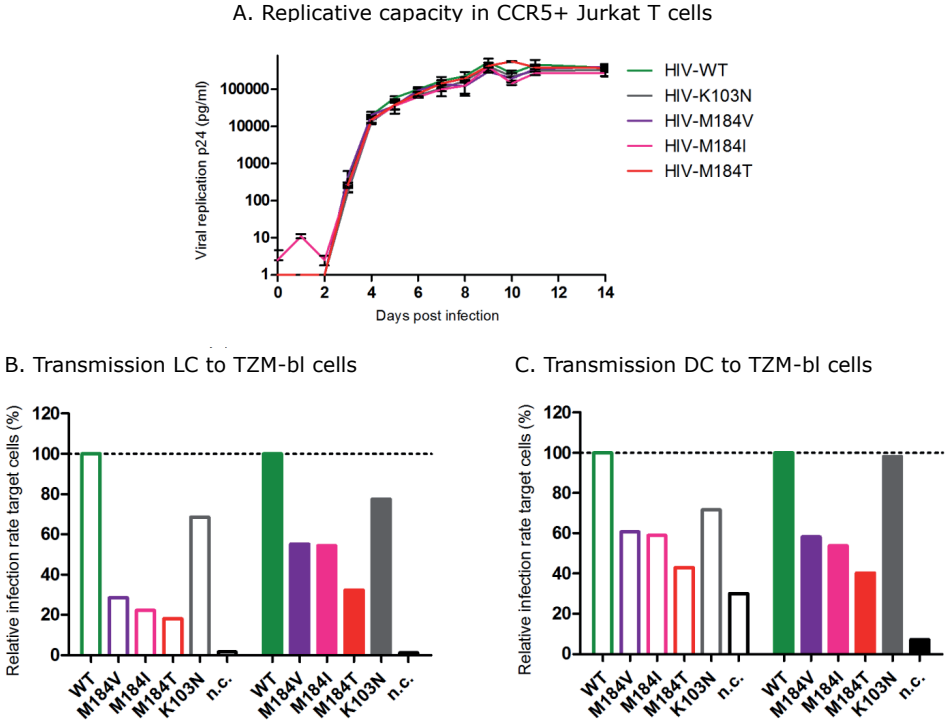


Figure 2. The diminished transmission of M184 variants is not caused by replication in target cells. A: To determine the replicative capacity of the virus panel in target cells, CCR5+ Jurkat T cells were infected in the absence of drugs and p24 production was monitored daily. Average infection with standard deviation is depicted. B-C: LCs (B) or DCs (C) were exposed to the equivalent of 17.5 (open bars) or 100 ng (closed bars) p24 for four days, extensively washed and co-cultured for two days with TZM-bl cells pre-incubated with indinavir. Infection was measured by luminescence compared to HIV-WT. Data are representative for 1 (LCs) and 2 (DCs) donors. Abbreviations: WT: HIV-WT, M184V: HIV-M184V, M184I: HIV-M184I, M184T: HIV-M184T, K103N: HIV-K103N, n.c.: no infection control.

Replicative capacity of mutant viruses in LCs and DCs

Next, we investigated if the observed differences in transmission efficacy can be explained by the RC of HIV-WT and the drug resistant viruses in LCs and DCs. To do so, LCs or DCs were exposed to all viruses for six days to enable *cis*-infection[25], after which the infection rate of CD1a (marker for DCs) positive cells was measured by detection of intracellular p24 by flow cytometry. It was previously described that LCs have a low susceptibility to HIV infection[20].

Although the percentage of infected LCs was indeed low, the level of infection by HIV-M184 variants was clearly reduced as compared to infection by HIV-WT. In agreement with the LC transmission experiments, the infection level of LCs by HIV-K03N was intermediate (Figure 3A). Furthermore, the level of infection of DCs was also in line with the observed transmission efficacy of DCs to T cells. Compared to HIV-WT, the infection rate of HIV-M184 variants was lower in DCs. The infection rate of HIV-K103N was comparable to HIV-WT, which is in agreement with the observed transmission data (Figure 3B). Combined, these data strongly indicate that the observed diminished transmission efficacy of HIV-M184 variants is caused by a decreased infection of LCs and DCs.

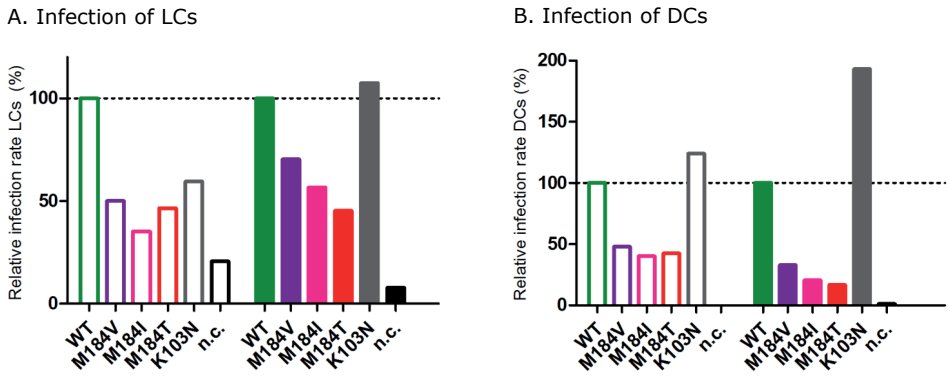


Figure 3. Lower infection of LCs and DCs by M184 variants. HIV infection of LCs (A) or DCs (B) by a panel of HIV-1 drug resistance variants (M184V, -I, -T and K103N) was measured after six days. All infections were started with virus equivalent to 17.5 ng (open bars) and 100 ng (filled bars) p24. Cells were stained with antibodies against CD1a and intracellular p24, infection was analyzed by flow cytometry. A representative donor for 2 (LCs) or 4 (DCs) independent experiments is shown. The relative percentage of p24 positive LCs compared to the percentage of cells infected by HIV-WT is depicted. Abbreviations: WT: HIV-WT, M184V: HIV-M184V, M184I: HIV-M184I, M184T: HIV-M184T, K103N: HIV-K103N, n.c.: no infection control.

Discussion

We have investigated the impact of drug resistance mutations in HIV RT on the transmission efficacy. K103N and M184V are both frequently observed in patients experiencing therapy failure, but whereas M184V is rarely detected in newly diagnosed patients, K103N is often observed[2, 7]. It was debated whether a diminished transmission efficacy could contribute to this observed discrepancy in prevalence. We compared transmission of wild type HIV with HIV-M184V in an HIV transmission model containing primary human DCs. In addition, we investigated transmission of HIV-K103N and HIV-M184T as controls. We

demonstrated that transmission by LCs and DCs to T cells is affected by the replication capacity defect caused by the M184 mutation.

It has been known for a long time that the RC of HIV harbouring M184V/I/T is reduced in primary T cells[9, 22]. Our data demonstrate that replication in primary LCs and DCs is also affected and as a result, transmission to T cells is diminished. Our results are in line with a study that compared transmission of SHIV wild type and M184V in rhesus macaques. In a repeated low-dose rectal transmission model, a larger inoculum was needed to successfully infect macaques with a SHIV variant containing M184V, indicating that mucosal transmissibility of the M184V variant is lower than wild type[13].

Previous studies have demonstrated that K103N has a modest impact on RC[10, 11]. In our HIV transmission model, the infection of and transmission by primary DCs of HIV-K103N was indeed consistently higher than the HIV-M184 variants. Remarkably, the level of infection of DCs by the K103N mutant was even higher than HIV-WT in the majority of donors. DCs obtained from one donor efficiently transmitted all viral variants except HIV-M184T (data not shown). The atypical transmission by one donor affirms that DCs have a major impact on the observations in the used assay. Interestingly, infection and transmission of HIV-K103N by DCs was even more efficient than HIV-WT transmission. It will be informative to investigate this observation in more detail as it may provide additional insights in the role of RC in transmission efficacy.

Several studies have addressed transmission efficacy in humans by comparing the prevalence of drug-resistant HIV variants in newly diagnosed patients and treatment-experienced patients[14, 26, 27]. These studies observed a reduced transmission rate of HIV variants harbouring drug resistance mutations. Such *in vivo* approaches measure the net result of potential differences in transmission efficacy combined with potential reversion of drug-resistance mutations after transmission to the new host. M184V is known to revert rapidly after transmission[12]. Using *in vitro* experiments, we were able to exclusively investigate the impact of drug resistance mutations on the transmission efficacy. Human DCs can either be productively infected in *cis*, or transfer virions by *trans* infection[28-30]. As the RC can only affect infection in *cis*, we used a human transmission model with a strong bias to *cis* infection.

Our data indicate that the RC of HIV variants with RT drug resistance mutations can impact the transmission efficacy. This may contribute to the discrepancy of the prevalence of M184V in treatment-experienced and naive individuals. In addition to RT drug resistance mutations, also variants harbouring protease or integrase inhibitor resistance mutations decrease viral RC[31, 32]. Determination of the impact of mutations affecting other steps in the viral replication cycle on transmission efficacy may enhance our understanding of the role of RC in transmission.

Conclusions

We have shown a diminished transmission of M184 variants from LCs and DCs to target cells, which was likely caused by the lower RC of M184 variants in LCs and DCs. Therefore, a diminished transmission efficacy of drug-resistant variants provides an additional mechanism explaining the observed discrepancy in prevalence of replication-deficient drug-resistant HIV variants in treatment-experienced and naive individuals.

Materials and Methods

Virus panel

The site-directed mutants M184V, M184I and M184T were previously generated[9, 22] in the background of HXB2. The mutation resulting in amino acid change K103N was introduced in HXB2 by site-directed mutagenesis using the previously described vector system with addition of primer K103N (5'-GTTACTGATTTGTTCTTTTTTAACCC-'3)[33]. Tropism of all viral variants was changed from CXCR4-tropic to CCR5-tropic by replacing the HXB2-V3 loop with the V3 loop of the CCR5-tropic lab strain BaL). HXB2-cBaL, referred to as HIV-WT, was generated by introducing a unique *BmgBI*-restriction site at position 7091 in HXB2. After restriction with BmgBI and NheI, nucleotides 7091 to 7260 of HXB2 were replaced by V3 of BaL. Subsequently, the plasmids containing drug-resistance mutations were restricted by NcoI and NheI and nucleotides 5675 to 7260 of these plasmids were replaced by the corresponding region of HXB2-cBaL.

Virus was obtained by transfection of HEK293T cells with plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Virus was quantified by p24 analysis. To exclude a possible influence of different batches, all steps of virus production were performed synchronized.

Cells

$CCR5^+$ Jurkat T cells were generated and maintained as previously described[20]. TZM-bl cells that express CCR5 were obtained through the NIH AIDS Research and Reference Reagent Program and maintained as recommended. Donor PBMCs were obtained by Ficoll-Paque density gradient centrifugation of heparinized blood from five HIV-seronegative donors, pooled and stored at -80°C until use. PBMCs were stimulated for three days with phytohaemagglutinin (2mg/l) in culture medium (RPMI 1640 with L-glutamine (Lonza, Verviers, Belgium), 10% fetal bovine serum (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 10 µg/ml gentamicin (Invitrogen, Breda, the Netherlands). LCs and DCs were obtained from multiple donors as previously described[20]. In short, for LC isolation, epidermis was separated by dipase II treatment (1mg/ml, Roche Diagnostic Systems, Somervolle, NJ) and cultured until LC maturation and migration. The

resulting cell suspension was purified by CD1a positive selection using MACS, according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). This procedure yielded a >95% pure CD1a LC population[23]. Monocytes were isolated by density centrifugation of PBMCs and cultured for five days in the presence of 800 U/ml IL-4 and 1000 U/ml GM-CSF to stimulate differentiation into DCs. The purity of obtained DCs was >90%[24].

Viral replication capacity

RC in $CCR5^+$ Jurkat T cells was determined in duplicate by infecting $2x10^6$ cells with the equivalent of 40 ng p24 of each virus. After two hours of inoculation, cells were washed twice with RPMI 1640 medium with L-glutamine and resuspended in 10 ml culture medium with 5 U/ml IL-2 (Roche, Mannheim, Germany). 300 µl cell-free supernatant was harvested daily for p24 analysis. Cultures were maintained for 14 days.

Infection of DCs and LCs was determined by infecting 50,000 cells with 17.5 or 100 ng p24. After 6 days of infection, cells were stained with α-DC1a as a marker for DCs and α-p24 as marker for productive HIV infection. Living cells were gated based on forward and sideward scatter; DCs were distinguished based on CD1a expression.

HIV transmission

5,000 DCs or LCs were infected with the equivalent of 17.5 and 100 ng p24 of all HIV variants for four to five days. After extensive washing, DCs or LCs were added to the target cells, which were either 50,000 $CCR5^+$ Jurkat T cells or TZM-bl cells that were pre-seeded on 96 well plate (confluence 70%). After 2 days of co-culture, infection was measured by flow cytometry as described above ($CCR5^+$ Jurkat T cells) or by luminescence (TZM-bl cells). TZM-bl cells were (pre-) incubated with 1,000 nM indinavir to investigate a single replication cycle after transmission.

Antibodies

The following antibodies were used: CD1a-FITC (BD Pharmingen, San Diego, CA, USA), CD1a-APC, CD3-PE (both BD Bioscience, San Jose, CA, USA), CD4-PerCP (BD Pharmingen), CD4-Alexa488 (Biolegend, San Diego, CA, USA), CCR5-APC (CD195) (BD Pharmingen), CXCR4-PerCP (R&D systems, Minneapolis, MN, USA), Langerin-PE (CD207), p24-PE (both Beckman Coulter, Fullerton, CA, USA)

Acknowledgements and funding

We thank Susan C. Aitken for critical reading of the manuscript. This study was supported by the Aids Fonds [grant number 2007034], the Netherlands Organization for Scientific Research [NWO VIDI grant 91796349 and VICI 91810619].

References

1. Wheeler WH, Ziebell RA, Zabina H et al. Prevalence of transmitted drug resistance associated mutations and HIV-1 subtypes in new HIV-1 diagnoses, U.S.-2006. *AIDS* 2010; **24**: 1203-12.
2. Vercauteren J, Wensing AM, van de Vijver DA et al. Transmission of drug-resistant HIV-1 is stabilizing in Europe. *J Infect Dis* 2009; **200**: 1503-8.
3. Wittkop L, Gunthard HF, de Wolf F et al. Effect of transmitted drug resistance on virological and immunological response to initial combination antiretroviral therapy for HIV (EuroCoord-CHAIN joint project): a European multicohort study. *Lancet Infect Dis* 2011; **11**: 363-71.
4. Kuritzkes DR, Lalama CM, Ribaudo HJ et al. Preexisting resistance to nonnucleoside reverse-transcriptase inhibitors predicts virologic failure of an efavirenz-based regimen in treatment-naive HIV-1-infected subjects. *J Infect Dis* 2008; **197**: 867-70.
5. Wainberg MA, Moisi D, Oliveira M et al. Transmission dynamics of the M184V drug resistance mutation in primary HIV infection. *J Antimicrob Chemother* 2011; **66**: 2346-9.
6. Richman DD, Morton SC, Wrin T et al. The prevalence of antiretroviral drug resistance in the United States. *AIDS* 2004; **18**: 1393-401.
7. van de Vijver DA, Wensing AM, Asjo B et al. HIV-1 drug-resistance patterns among patients on failing treatment in a large number of European countries. *Acta Dermatovenerol Alp Panonica Adriat* 2010; **19**: 3-9.
8. Schuurman R, Nijhuis M, van Leeuwen R et al. Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). *J Infect Dis* 1995; **171**: 1411-9.
9. Back NK, Nijhuis M, Keulen W et al. Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. *EMBO J* 1996; **15**: 4040-9.
10. Armstrong KL, Lee TH, Essex M. Replicative fitness costs of nonnucleoside reverse transcriptase inhibitor drug resistance mutations on HIV subtype C. *Antimicrob Agents Chemother* 2011; **55**: 2146-53.
11. Xu HT, Oliveira M, Quan Y et al. Differential impact of the HIV-1 non-nucleoside reverse transcriptase inhibitor mutations K103N and M230L on viral replication and enzyme function. *J Antimicrob Chemother* 2010; **65**: 2291-9.
12. Pingen M, Nijhuis M, de Bruijn JA et al. Evolutionary pathways of transmitted drug-resistant HIV-1. *J Antimicrob Chemother* 2011; **66**: 1467-80.
13. Cong ME, Youngpairoj AS, Aung W et al. Generation and mucosal transmissibility of emtricitabine- and tenofovir-resistant SHIV162P3 mutants in macaques. *Virology* 2011; **412**: 435-40.
14. Leigh Brown AJ, Frost SD, Mathews WC et al. Transmission fitness of drug-resistant human immunodeficiency virus and the prevalence of resistance in the antiretroviral-treated population. *J Infect Dis* 2003; **187**: 683-6.
15. Miller CJ, Li Q, Abel K et al. Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. *J Virol* 2005; **79**: 9217-27.
16. Keele BF, Giorgi EE, Salazar-Gonzalez JF et al. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A* 2008; **105**: 7552-7.
17. Spira AI, Marx PA, Patterson BK et al. Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques. *J Exp Med* 1996; **183**: 215-25.

18. Hu J, Gardner MB, Miller CJ. Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. *J Virol* 2000; **74**: 6087-95.
19. Fahrbach KM, Barry SM, Anderson MR et al. Enhanced cellular responses and environmental sampling within inner foreskin explants: implications for the foreskin's role in HIV transmission. *Mucosal Immunol* 2010; **3**: 410-8.
20. de Witte L, Nabatov A, Pion M et al. Langerin is a natural barrier to HIV-1 transmission by Langerhans cells. *Nat Med* 2007; **13**: 367-71.
21. Geijtenbeek TB, Kwon DS, Torensma R et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* 2000; **100**: 587-97.
22. Keulen W, Back NK, van Wijk A et al. Initial appearance of the 184Ile variant in lamivudine-treated patients is caused by the mutational bias of human immunodeficiency virus type 1 reverse transcriptase. *J Virol* 1997; **71**: 3346-50.
23. de Jong MA, de Witte L, Oudhoff MJ et al. TNF-alpha and TLR agonists increase susceptibility to HIV-1 transmission by human Langerhans cells ex vivo. *J Clin Invest* 2008; **118**: 3440-52.
24. Romani N, Gruner S, Brang D et al. Proliferating dendritic cell progenitors in human blood. *J Exp Med* 1994; **180**: 83-93.
25. Turville SG, Santos JJ, Frank I et al. Immunodeficiency virus uptake, turnover, and 2-phase transfer in human dendritic cells. *Blood* 2004; **103**: 2170-9.
26. Yerly S, Jost S, Telenti A et al. Infrequent transmission of HIV-1 drug-resistant variants. *Antivir Ther* 2004; **9**: 375-84.
27. de Mendoza C, Rodriguez C, Corral A et al. Evidence for differences in the sexual transmission efficiency of HIV strains with distinct drug resistance genotypes. *Clin Infect Dis* 2004; **39**: 1231-8.
28. Kawamura T, Gulden FO, Sugaya M et al. R5 HIV productively infects Langerhans cells, and infection levels are regulated by compound CCR5 polymorphisms. *Proc Natl Acad Sci U S A* 2003; **100**: 8401-6.
29. Cavrois M, Neidleman J, Kreisberg JF et al. In vitro derived dendritic cells trans-infect CD4 T cells primarily with surface-bound HIV-1 virions. *PLoS Pathog* 2007; **3**: e4.
30. Ballweber L, Robinson B, Kreger A et al. Vaginal langerhans cells nonproductively transporting HIV-1 mediate infection of T cells. *J Virol* 2011; **85**: 13443-7.
31. Martinez-Picado J, Savara AV, Sutton L et al. Replicative fitness of protease inhibitor-resistant mutants of human immunodeficiency virus type 1. *J Virol* 1999; **73**: 3744-52.
32. Hu Z, Kuritzkes DR. Effect of raltegravir resistance mutations in HIV-1 integrase on viral fitness. *J Acquir Immune Defic Syndr* 2010; **55**: 148-55.
33. van Maarseveen NM, Huigen MC, de Jong D et al. A novel real-time PCR assay to determine relative replication capacity for HIV-1 protease variants and/or reverse transcriptase variants. *J Virol Methods* 2006; **133**: 185-94.

Chapter 3

Evolutionary Pathways of Transmitted Drug-resistant HIV-1

Marieke Pingen[1,2], Monique Nijhuis[1], Johan A. de Bruijn[1], Charles A.B. Boucher[2] and Annemarie M.J. Wensing[1]

[1] Department of Virology, Medical Microbiology, University Medical Center Utrecht, the Netherlands
[2] Department of Virology, Erasmus Medical Center, the Netherlands

Journal of Antimicrobial Chemotherapy, 2011; 66:1467-80.

Abstract

Several large studies in Europe and the United States revealed that approximately 10% of all newly diagnosed patients harbour HIV-1 variants with at least one major resistance-associated mutation. In this review we discuss the underlying mechanisms that drive evolution of drug-resistant viruses after transmission to the new host. In a comprehensive literature search 12 papers describing evolution of 58 cases of transmitted resistant HIV-1 variants were found.

Based on the observations in literature we propose three pathways describing the evolution of resistant HIV-1 after transmission to a new host. Firstly, reversion of the resistance mutation towards wild-type may rapidly occur when drug resistance mutations severely impact replicative capacity. Alternatively, a second pathway involves replacement of transmitted drug resistance mutations by atypical amino acids that also improve viral replication capacity. In the third evolutionary pathway the resistance mutations persist either because they do not significantly affect viral replication capacity or evolution is constrained by fixation through compensatory mutations.

In the near future ultra-sensitive resistance tests may provide more insight into the presence of archived and minority variants and their clinical relevance. Meanwhile, clinical guidelines advise to perform population sequence analysis of the baseline plasma sample to identify transmission of resistance. Given the limited sensitivity of this technique for minority populations and the delay between the moment of infection and time of analysis, knowledge on the described evolutionary mechanisms of transmitted drug resistance patterns is essential for clinical management and public health strategies.

Introduction

Current guidelines supporting the earlier start of therapy and the recent introduction of new classes of potent and more tolerable antiretroviral drugs have reduced the number of HIV-infected individuals with detectable plasma HIV-RNA in the developed world. Given the correlation between the level of HIV-RNA in the individual and the risk of transmission,[1] it may be presumed that increased viral suppression at a population level will reduce the number of new infections[2]. Consequently, the individuals most likely to transmit HIV will be as follows: those unaware of their infection status; diagnosed patients with high CD4 cell counts who are not yet eligible for therapy; or treatment-experienced patients with unsuppressed plasma viremia caused by therapy failure. The latter group represents an obvious risk for transmission of drug-resistant HIV; several reports have indicated that 75-80% of treated individuals with detectable plasma HIV RNA levels carry viruses with reduced susceptibility to one or more drugs[3, 4].

Surveillance studies have shown that approximately 10% of new HIV-1 infections involve drug-resistant strains, indicating that treated individuals are indeed involved in the spread of new infections. However, it has been shown that individuals *de novo* infected with drug-resistant viruses can also serve as a source of subsequent infections and thus contribute to the spread of drug-resistant HIV[5-7].

The European HIV-1 surveillance program Strategy to Control SPREAD of HIV Drug Resistance (SPREAD) is the largest ongoing prospective study investigating transmission of drug-resistant HIV. In this programme, data and samples from recently diagnosed treatment-naïve individuals are collected using a strategy enabling representative sampling among different transmission groups. The program has revealed a stabilizing prevalence of viruses with drug resistance mutations of 8.4% in 2793 treatment-naïve patients in 2002-05[8]. This level is comparable with the earlier retrospective European Combined Analysis of Resistance Transmission over time of Chronically and acute infected HIV patients (CATCH) study (10.4%), which was conducted from 1996 to 2002[9]. Similar results have been reported in a large study in the United States. Between 1997 and 2001 over 1,080 recently infected treatment-naive patients were enrolled of whom 8.3% carried HIV-1 with resistance associated mutations[10]. More recent data from patients diagnosed in 2006 in 11 surveillance areas in the United States showed a higher prevalence of 14.6%[11]. In studies conducted in specific risk groups, e.g. Caucasian homosexual men in large cities, even higher levels of transmitted resistance of up to 25% have been reported[12-14]. In resource-limited countries with a shorter history of antiretroviral availability, infection with drug-resistant HIV-1 is less common[15]. Nevertheless, a recent report has shown that even in these settings transmission of resistance can be relatively frequent if exposure to therapy in the area is high[16]. Furthermore, high rates of

mother-to-child transmission of drug-resistance have been reported, especially in studies investigating the effect of single-dose Nevirapine in resource limited settings[17]. Recently, interventions with expanded drug regimes including short courses of NRTIs or replacement of single-dose Nevirapine by a complete HAART regimen have shown decreased rates of transmission and resistance[18, 19].

Although information on follow-up of individuals with transmitted resistance is limited in literature, most available cases show persistence of major drug resistance profiles for a long time[20-29]. Nonetheless, reversion of the transmitted drug resistance patterns in the plasma has been reported as well[20-26, 28, 30, 31]. Furthermore, data from the CATCH study indicated a higher level of resistance in recently infected individuals compared with individuals with a longer or unknown duration of infection, suggesting that reversion of resistant viruses to drug sensitive variants does occur rather frequently over time[32]. Additional evidence of viral evolution following infection comes from the frequent detection of atypical variants on drug resistance positions, mainly 215 in reverse transcriptase (RT), in newly diagnosed individuals[30, 33, 34]. These mutations, often representing molecular intermediates between drug-resistant mutants and wild-type, are rarely seen in treated individuals.

Thus, in the absence of drug selective pressure in the new host transmitted drug-resistant viruses may revert to wild-type, evolve to other variants or persist. Insight into whether particular variants are likely to revert, evolve or persist has important implications for prevalence studies, public health and clinical management. This review discusses the current literature and will try to shed some light on the mechanisms that drive the evolution of resistant variants after transmission to a new host.

Methods

A comprehensive literature search using the primary search terms "reversion" or "persistence" combined with "HIV", "transmission" and "resistance" and on Pubmed resulted in the selection of a number of papers describing the evolution of transmitted resistance in the new host in absence of therapy[22, 24, 28, 29, 31, 35]. Some additional papers regarding the evolution of drug-resistant HIV-1 after transmission that were found through citations were also included[20, 21, 23, 25-27, 30].

Since it is only possible to distinguish between natural variation and transmitted resistance for mutations that are not present as variants in the natural quasispecies, this review focuses on primary mutations as listed in the International AIDS Society mutation table[36]. This list was chosen because it is used most often to characterise resistance-associated mutations. Secondary

mutations may also be selected under drug-selective pressure, but may also appear as natural polymorphisms and therefore cannot be used as reliable indicators of exposure to antiretroviral drugs in a previous host. Recently, an updated consensus list of the WHO genotypic definition of transmitted drug resistance was published, which will be useful for future publications[37].

In the literature, time after diagnosis or (presumed) infection is calculated in weeks or months. For convenience and transparency of the literature overview in Table 1, we calculated persistence over time in months. Described cases of superinfection[22] or a presumed recombination event[21] were excluded from this review. Several papers did not only investigate mutations present in virus particles in plasma, but also analysed peripheral blood mononuclear cells (PBMCs) to study archived mutations[24, 27] or performed clonal sequencing to look into the presence of viral subpopulations[24, 28, 31]. These data are included in the results section, but not in Table 1.

Results

A total of 58 cases infected with drug-resistant HIV-1 from 12 papers were included. An overview of these cases and the evolution of the particular mutational patterns over time are displayed in Table 1. These papers focused either on the reverse transcriptase (RT) domain or on both RT and protease (PR).

One of the first papers describing the evolution of transmitted resistance mutations in RT was published in a Swiss Cohort[30]. Yerly *et al* reported four seroconverters who were infected with viruses harbouring mutations in RT associated with resistance to thymidine analogues (TAMs). Over time, partial reversion of resistance was observed in all cases with persistence of the M41L only. On position 215 the resistant variants Phenylalanine and Tyrosine (F and Y, respectively) were replaced in plasma by other variants than wild-type. The Amsterdam cohort reported similar findings in eight patients infected with a virus containing TAMs[20].

Several other groups have reported on more extensive transmitted resistance[21, 22, 25, 26, 29, 31]. In general, in the absence of treatment, the RT mutation M184V became undetectable by population sequencing and 215 mutations are replaced by other variants. Within the extensive PR profiles, individual mutations occasionally reverted over time but the majority of mutations persisted.

In the San Diego cohort, multiple cases of transmitted Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI)-related resistance were followed during their treatment naive period[28]. The NNRTI resistance mutation K103N was seen in 11 men who have sex with men and reverted to wild-type in only two cases;

incomplete reversion was observed in two other patients after 38 and 212 weeks. Other NNRTI resistance mutations reverted only partially (Y181C and P225H) or persisted (Y188L) during follow-up. Some resistance mutations appeared in mixture during follow-up; these mutations might have been present on a level near the detection threshold of the assay.

Limited information is available on evolution of resistance in other genes. Besides the PR gene itself, the PR substrate Gag also plays an important role in the development of resistance against PR Inhibitors (PIs), but is not included in this review[38]. Polymorphisms and resistance-related mutations in Gag are not well defined yet, making it difficult to distinguish between transmitted drug resistance and natural variation. Of interest, a higher prevalence of specific Gag resistance mutations has been described in patients infected with viruses also harbouring PI mutations compared to patients infected with wild-type viruses[39]. Unfortunately, no follow-up data on evolution of these profiles have been reported. Also, for newer drug classes such as entry and integrase inhibitors, more information on the natural variation in their target genes is necessary to enable identification of indicators of transmitted resistance to new compounds. Two groups reported transmission of viruses with resistance mutations to the fusion inhibitor enfuvirtide[40, 41]. However, long-term persistence or reversion after transmission of variants resistant to this inhibitor has not yet been documented. Only one case of transmitted resistance against integrase inhibitors has been described so far. In this case integrase resistance mutations G140S and Q148H persisted for 48 weeks[42]. It would be of interest to investigate the evolution of transmitted resistance against integrase inhibitors more in depth.

Evolutionary Pathways of Transmitted Drug-resistance

After the complete removal of drug selection pressure in treated individuals, a rapid reappearance of wild-type HIV that persisted in the cellular compartment has been observed[43]. After transmission of drug-resistant HIV to a new host, such a rapid shift is unlikely given that wild-type virus is rarely co-transmitted[44]. Therefore, when only drug-resistant variants are present in the new host, a novel starting point for viral evolution is created; there is no 'memory' of the original wild-type in the quasispecies. Nucleotide changes in the quasispecies are modulated by chance events and will be selected if they have a beneficial effect on viral fitness. Transmitted drug-resistant variants may persist or fade away from detection in the plasma depending on their relative fitness in the new environment.

As summarized in Table 1, particular transmitted drug resistance mutations or patterns seem to persist in the plasma quasispecies while others are lost over time. We identified three possible evolutionary pathways of viral evolution after transmission, caused by different underlying mechanisms.

Pathway I: Evolution to wild-type (Reversion of Transmitted Drug Resistance)

It is widely accepted that most major drug resistance mutations in RT and PR lower the replicative capacity (RC) of HIV[45-47]. If there is no beneficial effect of the resistance mutations after transmission to a new host, reversion to wild-type may be observed. This pathway is graphically depicted in Figure 1.

A striking example from the reviewed literature as summarised in Table 1 is the NRTI resistance associated mutation M184V, which was replaced by wild-type in the plasma in six out of seven cases within 16 months (median 9.5 months; range 5-15 months). This mutation persisted in only one case, together with a combination of RT mutations (M41L, M184V, T215Y, K219G in RT) in a virus also harbouring multiple PI mutations[25]. The M184V significantly affects the RC of HIV-1[48]. Since the difference between wild-type and the drug-resistant variant is only one nucleotide change ($ATG_{WT} \rightarrow GTG_{var}$), a virus containing only the M184V mutation can rapidly revert to more replication competent wild-type virus.

Incomplete reversion can be observed after transmission of resistance patterns that require more than one mutation for full reversion to wild-type. This is commonly observed at particular amino acid positions where two nucleotide changes are necessary to revert from the mutant form to wild-type incomplete reversion may result in intermediates. Such intermediates or revertants are commonly observed at codon 215 in RT and codon 82 in PR. The T215Y and T215F

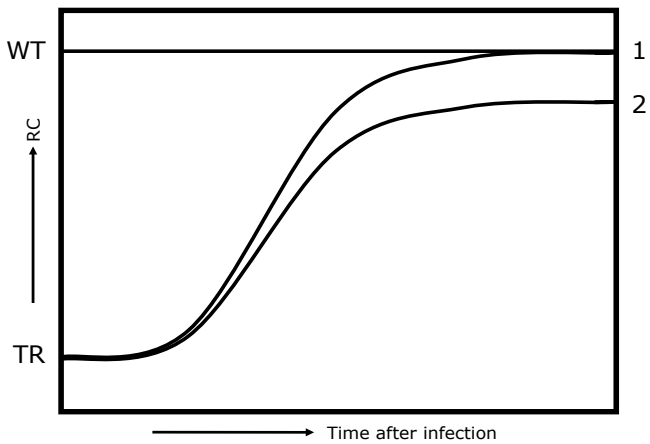


Figure 1. Evolution to wild-type. After transmission, the RC (y-axis) of the transmitted drug-resistant variant (TR) is lower than that of wild-type (WT). Due to complete (1) or incomplete (2) reversion of the drug resistance mutation, the replicative capacity is restored or improved.

Table 1. Evolution of major transmitted resistance mutations in plasma viral RNA.

Patient ID	Months after infection or diagnosis	Resistance mutations		Pathway			First author (ref)
		Reverse transcriptase	Protease	Reversion	Atypical variants	Persistence	
Profiles containing only NRTI-related mutations							
H	**0, 28**	**M41L**	**-**			**M41L**	**Pao [26]**
I	**0** 2	**A62V** -	**-**	 A62V→wt			**Pao [26]**
B	**0, 15**	**T69N**	**-**			**T69N**	**Pao [26]**
G	**0, 32**	**T69N**	**-**			**T69N**	**Pao [26]**
M	**0, 16**	**T69N**	**-**			**T69N**	**Pao [26]**
8	**0** 36	**K70R** -	**nd**	 K70R→wt			**de Ronde [20]**
B	**0** 6 12	**T215F** T215L T215L/F	**nd**		 T215F→L T215L→L/F		**Yerly [30]**
E	**0, 6** 12, 16, 18	**T215Y** T215C	**nd**		 T215Y→C		**Yerly [3]**
3	**0** 6 12	T215Y T215Y/S T215S	nd	 T215Y→Y/S T215Y/S→S			de Ronde [20]
K	**0, 11**	**T215D**	**-**			**T215D**	**Pao [26]**
N	**0, 13**	**T215D**	**-**			**T215D**	**Pao [26]**
J	**0** 9, 22, 36	**K219Q** -		 K219Q→wt			**Pao [26]**
35	**0** 6 12 24	**M41L, T215Y** M41L, T215Y/C M41L, T215C M41L, T215C	-		 T215Y→Y/C T215Y/C→C	 M41L	**Ghosn [27]**

Table 1. Continued

Patient ID	Months after infection or diagnosis	Resistance mutations		Pathway			First author (ref)
		Reverse transcriptase	Protease	Reversion	Atypical variants	Persistence	
E	**0** 21 33	**M41L, T215Y** M41L, T215C M41L, T215C	**-**		 T215Y→C 	 M41L	**Pao [26]**
G	**0** 42	**M41L, T215Y** M41L, T215D	**nd**		 T215Y→D	 M41L	**Yerly [30]**
1	**0** 6, 12 18 24, 30, 36	**M41L, T215Y** M41L, T215Y/N M41L, T215N M41L, T215N/D	**nd**	 T215Y→Y/N T215Y/N→N T215N→N	 T215N→D	 M41L	**de Ronde [20]**
2	**0** 6 12	**M41L, T215Y** M41L, T215Y/N M41L, T215N/D	**nd**	 T215Y→Y/N T215Y/N→N	 T215Y/N→D	 M41L	**de Ronde [20]**
4	**0** 6 12, 18, 24 30	**M41L, T215Y** M41L, T215D M41L, T215D/S M41L, T215D/S	**nd**	 T215D→S	 T215Y→D T215D→D 	 M41L	**de Ronde [20]**
7	**0, 6**	**M41L, T215D**	**nd**			**M41L, T215D**	**de Ronde [20]**
C	**0, 7**	**M41L, T215L**				**M41L, T215L**	**Pao [26]**
34	**0, 24**	**M41L, L210W, T215C**				**M41L, L210W, T215C**	**Ghosn [27]**
5	**0** 6	**D67N, K70R, T215F** D67N/D, K70R, T215S/L	**nd**	 D67N→N/D, T215FS	 T215F→L	 K70R	**de Ronde [20]**
6	**0, 6**	**D67N, K70R, K219Q**	**nd**			**D67N, K70R, K219Q**	**de Ronde [20]**

Table 1. Continued

Patient ID	Months after infection or diagnosis	Resistance mutations		Pathway			First author (ref)
		Reverse transcriptase	Protease	Reversion	Atypical variants	Persistence	
B	**0**	**D67N/D, K70K/R,** K219G	**-**				**Barbour [25]**
	6	K70K/R, K219G		D67D/N→wt			
	15	K70R, K219G				K70R, K219G	
F	**0**	**D67N, K70R,**	**nd**				**Yerly [30]**
	46, 58	T215F, K219Q		D67N, K70R, T215F, K219Q→wt			
		-					
Profiles containing only NNRTI-related mutations							
36	**0, 24**	**K103N**	**-**			**K103N**	**Ghosn [27]**
01-0125	**3, 4**	**K103N**	**-**			**K103N**	**Little [28]**
01-0143	**1.5, 2.5, 4, 5, 6, 6.5**	**K103N**	**-**			**K103N**	**Little [28]**
01-0566	**3, 6, 9, 13, 16.5, 22, 25.5, 27, 29.5, 34**	**K103N**	**-**			**K103N**	**Little [28]**
01-0183	**3, 4, 5**	**K103N**	**-**			**K103N**	**Little [28]**
01-0503	**1, 1.5, 3.5, 5.5, 8.5, 10.5, 13.5**	**K103N**	**-**			**K103N**	**Little [28]**
01-0180	**3, 4, 5, 6**	**K103N**	**-**				**Little [28]**
	6.5, 7.5, 8.5, 9.5, 10.5, 13.5, 17, 19.5, 23.5, 23.5, 27.5, 31	K103K/N		K103N→K103K/N			
	34, 37, 39.5, 42.5	-		K103K/N→wt			

Table 1. Continued

Patient ID	Months after infection or diagnosis	Resistance mutations		Pathway			First author (ref)
		Reverse transcriptase	Protease	Reversion	Atypical variants	Persistence	
01-0512	**2, 2.5, 5, 6.5, 8.5, 11, 15, 18, 21, 25.5, 28.5, 36**	**K103N**	**-**				**Little[28]**
	31, 34, 38.5, 41,5	K103N, P225P/H			+ P225P/H		
	49	K103K/N		P225P/H→wt, K103N→K/N			
A	**0**	**Y181C**					**Pao [26]**
	25	-		Y181C→wt			
2	**0, 5**	**G190A**	**-**			**G190A**	**Chan [23]**
Patient F	**0**	**K103N, V108I/V**					**Pao [26]**
	23	K103N		V108I→wt		K103N	
01-0182	**3, 4.5, 5, 7.5**	**K103N, Y181C**	**-**				**Little [28]**
	8.5, 10.5	K103K/N, Y181Y/C, G190G/A		K103N→K/N, Y181C→Y/C	+ G190A/G		
Profiles containing both NRTI- and NNRTI-related mutations							
01-0559	**1, 3.5, 6, 10, 11.5, 14, 18, 22, 25.5, 35**	**K103N, P225H**	**-**				**Little [28]**
	30.5	K103N, K219K/R, P225H		K219K/R→wt,	+ K219K/R		
	39.5	K103N, P225P/H		P225H→P/H		K103N	

Table 1. Continued

Patient ID	Months after infection or diagnosis	Resistance mutations		Pathway			First author (ref)
		Reverse transcriptase	Protease	Reversion	Atypical variants	Persistence	
Profiles containing both NRTI- and NNRTI-related mutations							
01-0629	**1.5**	**D67N, K103K/N, M184M/V, T215S, K219E**	**-**				**Little [28]**
	5, 7.5, 9.5, 13, 16.5, 21, 26, 29, 31.5, 37, 40	D67N, T215S, K219E		M184M/V, K103K/N→wt		D67N, T215S, K219E	
Profile containing only PI related mutation							
2	**0, 10**		**M46I**			**M46I**	**Polilli [29]**
Profiles containing both RT- and PI-related mutations							
33	**0**	**M41L, T215Y**	**I84V, L90M**				**Ghosn [27]**
	6	M41L, T215Y	I84V, L90M				
	12	M41L, T215Y/C	I84V, L90M		T215Y→Y/C		
	24	M41L, T215C	I84V, L90M		T215Y/C→C		
	36	M41L, T215C	I84V, L90M				
	48	M41L, T215C	I84V, L90M			M41L, I84V, L90M	
C	**0, 9**	**M41L, M184V, T215Y**	**M46I, I54M, V82A, L90M**			**M41L, M184V, T215Y, M46I, I54M, V82A, L90M**	**Barbour [25]**
1	**0, 2**	**M41L, L210W, T215C**	**V82A, L90M**				**Chan [23]**
	5	M41L, E44D, M184V, L210W, T215C	V82A, L90M		+ E44D, M184V		

Table 1. Continued

Patient ID	Months after infection or diagnosis	Resistance mutations		Pathway			First author (ref)
		Reverse transcriptase	Protease	Reversion	Atypical variants	Persistence	
	9	M41L, E44D/E, M184M/V, L210W, T215C	V82A, L90M	E44D→D/E			
	12	M41L, E44D, M184M/V, L210W, T215C	V82A, L90M	E44D/E→D			
	14	M41L, E44D/E, L210W, T215C	V82A, L90M	M184V→wt, E44D→D/E			
	16	M41L, E44D, L210W, T215C	V82A, L90M	E44D/E→D			
	19	M41L, E44D, L210W, T215C	V82A, L90M				
	22	M41L, E44D/E, L210W, T215C	V82A, L90M	E44D→D/E			
	25	M41L, L210W, T215C	V82A, L90M				
	26	M41L, E44D, L210W, T215C	V82A, L90M				
	29	M41L, E44D/E, L210W, T215C	V82A, L90M	E44D/E→D			
	32	M41L, E44D/E, L210W, T215S	V82A, L90M	T215C→S		M41L, L210W, V82A, L90M	
D	**0, 12**	**M41L, 210W, T215C**	**D30N, M46L**			**M41L, L210W, T215C, D30N, M46L**	**Barbour [25]**

Table 1. Continued

Patient ID	Months after infection or diagnosis	Resistance mutations			Pathway		First author (ref)
		Reverse transcriptase	Protease	Reversion	Atypical variants	Persistence	
01-0449	**3, 4, 6, 8, 10, 12, 15.5, 23**	**M41L, D67N, K70R, T215Y, K219Q**	**D30N, M46I, I84V, L90M**				**Little [28]**
	14, 20, 31, 43.5	M41L, D67N, K70R/K, T215Y, K219Q	D30N, M46I, I84V, L90M				
	27, 35, 37, 46	M41L, D67N, T215Y, K219Q	D30N, M46I, I84V, L90M	K70R→wt			
	49, 52	M41L, D67N, T215Y/C, K219Q	D30N, M46I, I84V, L90M		T215Y→Y/C	M41L, D67N, K219Q, D30N, M46I, I84V, L90M	
01-0575	**3, 6, 8.5**	**M41L, L74V, M184V, L210W, T215Y**	**M46I, V82A**				**Little [28]**
	11.5	M41L, L74V, M184M/V, L210W, T215Y	M46I, V82A				
	15, 18.5	M41L, L74V, L210W, T215Y, K219K/R	M46I, V82A	M184V→wt	+ K219R/K		
	22.5	M41L, L74V, L210W, T215C/S	M46I, V82A	K219R/K→wt, T215Y→S	T215Y→C		
	25.5, 28	M41L, L74V, L210W, T215C/S	M46I, V82A/V	V82A→A/V			
	30.5	M41L, L74V, Y181Y/C, L210W, T215C/S	M46I, V82A	V82A/V→A	+ Y181Y/C		
	33, 35	M41L, L74V, Y181Y/C, L210W, T215C/S	M46I, V82A/V	V82A→A/V		M41L, L74V, Y181Y/C, L210W, M46I	
	39	M41L, L74V, Y181Y/C, L210W, T215S	M46I, V82A/V	T215C/S→S			

Table 1. Continued

Patient ID	Months after infection or diagnosis	Resistance mutations		Pathway			First author (ref)
		Reverse transcriptase	Protease	Reversion	Atypical variants	Persistence	
PHI 3	**3, 4, 6.5**	**K103N**	**I54V, V82T, I84V, L90M**				**Brenner [21]**
	7.5, 8.5	K103N		I84V→wt		K103N, I54L, V82T, L90M	
PHI-3	**2, 3, 5, 8, 9, 39**	**K103N**	**I54V, V82T, I84V, L90M**			**K103N, I54V, V82T, I84V, L90M**	**Brenner [22]**
PHI 2	**2**	**M41L, K103N, M184V, T215Y**	**G48V, V82A, L90M**				**Brenner [21]**
	3	M41L, K103N, M184V, T215Y	G48V/G, V82A L90M				
	5.5, 6.5	M41L, K103N, M184V, T215Y	V82A, L90M	G48V→wt			
	7.5	M41L, K103N, M184V/M, T215Y	V82A, L90M	M184V→V/M			
	8.5	M41L, K103N, T215Y	V82A, L90M	M184V/M→wt			
	11	M41L, K103N, T215C	V82A, L90M		T215Y→C	M41L, K103N, V82A, L90M	
PHI-2	**2, 3, 6, 7, 8, 9**	**M41L, K103N, M184V, T215Y**	**G48V, V82A, L90M**				**Brenner [22]**
	12	M41L, K103N, T215Y	V82A, L90M	M184V, G48V→wt		M41L, K103N, T215Y, V82A, L90M	
L	**0, 18**	**T69N, Y188L, K219Q**	**I54L, I84V, L90M**			**T69N, Y188L, K219Q, I54L, I84V, L90M**	**Pao [26]**

Table 1. Continued

Patient ID	Months after infection or diagnosis	Resistance mutations		Pathway			First author (ref)
		Reverse transcriptase	Protease	Reversion	Atypical variants	Persistence	
32	**0, 6**	**M41L, K103N, L210W, T215Y**	**L90M**				**Ghosn [27]**
	12	M41L, K103N, L210W, T215Y/C	L90M		T215Y→Y/C T215Y/C→C		
	24	M41L, K103N, L210W, T215C	L90M			M41L, K103N, L210W, L90M	
01-0507	**1.5, 2.5, 3.5, 5.5**	**M41L, D67N, K103N, T215Y**	**I84V, L90M**				**Little [28]**
	7, 8	M41M/L, D67N, K103N, T215C/Y	I84V, L90M				
	9.5	M41L, D67N, K103N, T215C/Y	I84V, L90M	M41L→L/M, D67N→D/N	T215Y→C/D/G/Y	K103N, I84V, L90M	
	14, 16	M41L/M, D67D/N, K103N, T215C/D/G/Y	I84V, L90M				
	15	M41L, K103N, T215C	M46L, I54V, V82A, L90M		T215Y/C→C	M41L, K103N, M46L, I54V, V82A, L90M	
B	**0** 24, 35	**D67N, T69D, K103N, Y181C, T215L**	**M46I, L90M L90M**				**Delaugerre [24]**
		D67N, T69D, K103N, Y181C, T215L		M46I→wt		D67N, T69D, K103N, Y181C, T215L, L90M	
01-0483	**3, 3.5**	**M41L, V75I, Y188L, T215L, K219E**	**D30N**			**M41L, V75I, Y188L, T215L, K219E, D30N**	**Little [28]**

Table 1. Continued

Patient ID	Months after infection or diagnosis	Resistance mutations		Pathway			First author (ref)
		Reverse transcriptase	Protease	Reversion	Atypical variants	Persistence	
A	**0**	**M41L, K103N, M184V, L210W, T215Y**	**M46L, I54V, V82A, L90M**				**Barbour [25]**
	6	M41L, K103N, M184V/M, L210W, T215Y	M46L, I54V, V82A, L90M				
	10	M41L, K103N, L210W, T215Y	M46L, I54V, V82A, L90M	M184V→wt			
	12	M41L, K103N, L210W, T215Y/C	M46L, I54V, V82A, L90M		T215Y→Y/C		
	15	M41L, K103N, T215C	M46L, I54V, V82A, L90M		T215Y/C→C	M41L, K103N, M46L, I54V, V82A, L90M	
D	**0, 1, 9, 17**	**M41L, E44D, K103N, L210W, T215Y, K219R**	**I54V, V82A, L90M**			**M41L, E44D, K103N, L210W, T215Y, K219R, I54V, V82A, L90M**	**Pao [26]**
index patient	2	**E44D, D67N, L74V, Y181V, G190S, T215D**	**V82T**				**Neifer [25]**
	19	E44D/E, D67N/D, L74V, Y181V, G190S, T215D	V82T	E44D→E/D, D67N→N/D			
	28	L74V/L, Y181V, G190S, T215D	V82T	E44E/D, D67N→wt L74V→V/L, V82T→A	Y181V, G190S, T215D,		

Data is ordered based on mutational profile, divided based on related drug class (NRTI, NNRTI, PR) and complexity of profile. All results are obtained using population sequencing; where other methods are used concurrently, this is described in the text. The first detected profile is in bold, evolutionary patterns are in normal characters. In case a mixture of more pathways is observed, the mutation is depicted in all relevant columns.

mutations are selected by thymidine analogues and cause (cross) resistance to all NRTIs[20, 33]. Both changes have a considerable impact on RC[49]. It appears that the intermediate variants T215S, T215N and T215I have considerably higher replication capacity than T215Y and T215F, almost approaching the RC of wild-type[20]. As a result, these intermediate variants tend to persist, as evident in Table 1. Of the 16 patients infected with a viral variant containing either the T215Y or 215F mutation, five were replaced with intermediates that persisted in four cases.

Pathway II: Evolution towards Atypical Variants

After transmission of drug-resistant HIV to a new host, a novel amino acid may be selected that is neither the wild-type codon nor an intermediate towards wild-type. In general, these atypical variants confer a higher replication capacity than the originally transmitted drug-resistant amino acid (Figure 2). In some occasions, these atypical variants represent amino acids that have been encountered in the untreated population and have been described as natural polymorphisms.

This phenomenon is often observed at position 215 in RT. At this position not only revertants are observed (as described above) but also variants that are not obvious intermediates between resistant variants and wild-type. Like revertants, these atypical variants have an increased replication capacity and increased susceptibility for thymidine analogues as compared to the originally transmitted drug-resistant variant. Figure 3 provides an overview of all the identified variations and their possible evolutionary

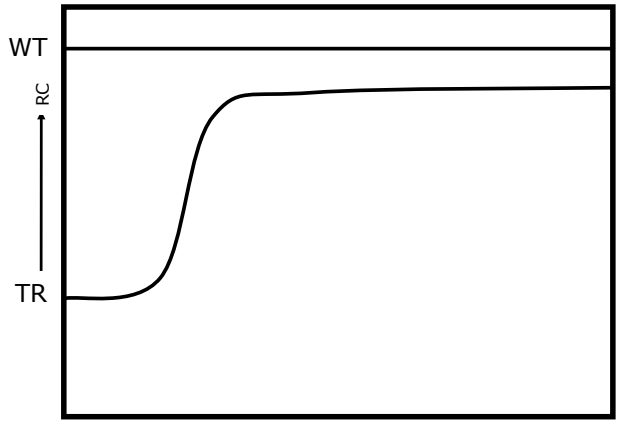


Figure 2. Selection of atypical mutations. After transmission of a drug-resistant HIV variant, atypical amino acids that are neither wild-type nor intermediates may be selected, leading to improved RC.

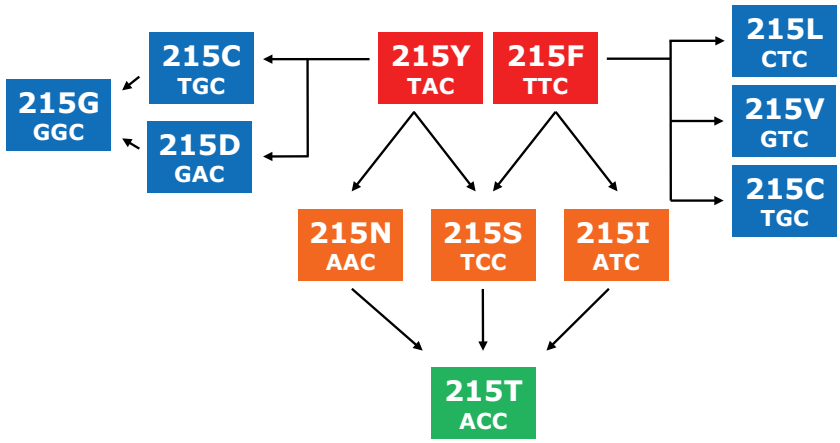


Figure 3. Evolution of transmitted variants at position 215 of RT. Variants T215Y and T215F (depicted in red) cause resistance to NRTIs. 215S/N/I (orange) are intermediates between resistant and wild-type, and 215C/D/L/V/C/G (blue) are atypical variants. The wild-type amino acid on position 215 is Threonine (T) (depicted in green)

Selection of a particular pathway may depend on the genetic variation in the drug-resistant variant. For instance, Garcia-Lerma *et al.* found that if the TAM M41L or L210W was present, the atypical variants T215D and T215C were observed more frequently than the intermediate T215S[33]. However, when changes at codon 215 were present in isolation the T215S was selected more often. We observed a similar trend in the reviewed literature for T215D and C, but not for T215S, possibly because this mutation was selected in only 4 cases.

Interestingly, data from the World-wide Analysis of resistance Transmission over time of Chronically and acute infected HIV patients (WATCH) study indicate that novel variants at resistance positions are more often observed in newly diagnosed patients infected with other drug-resistant changes compared to patients infected with a wild-type background[16]. This observation provides further evidence that they develop from resistant variants rather than from natural variation of wild-type.

Pathway III: Persistence of Transmitted Drug Resistance

Three mechanisms can explain persistence of resistant variants in the new untreated host.

Persistence because of a minimal reduction in RC

If the RC of transmitted drug-resistant variants is almost equal to that of wild-type, the replicative advantage of wild-type is limited and mutations may persist for a considerable time (Figure 4). For example, NNRTI resistance mutations, particularly

K103N, have only a moderate effect on RC[50]. In the reports included in this review, transmission of K103N-containing variants was observed 21 times and persisted completely in 17 out of 21 patients for a median duration of 16 months (Table 1). Most other NNRTI resistance mutations also seem to be able to persist. In addition, the NRTI associated mutations L210W and the K219R/G/Q/E, which have also been reported to exert little effect on RC also persisted in most cases[49].

Figure 4. Persistence because of a minimal reduction in RC. If the RC of the resistant variant (almost) equals the RC of wild-type, persistence may occur for a considerable time.

Persistence because reversion is prohibited by compensatory fixation

Interestingly, there are several examples of resistant viruses with an RC significantly lower than that of wild-type persisting after transmission to a new host. We proposed a mechanism called compensatory fixation to explain persistence of combinations of PI-resistant mutations in patients interrupting their PI therapy[51, 52].

In the treated source, compensatory mutations may appear after the initial selection of drug resistance mutations that lower the RC. After transmission to a new host, evolution may be expected to occur in a stepwise manner. However, if all possible nucleotide changes would initially decrease the RC, reversion to wild-type will be blocked (Figure 5).

We propose that this mechanism may help to explain persistence of some transmitted drug-resistant genotypes. Table 1 shows that variants with PR-associated resistance mutations generally tend to persist. Persisting profiles are often characterized by the presence of several major protease resistance

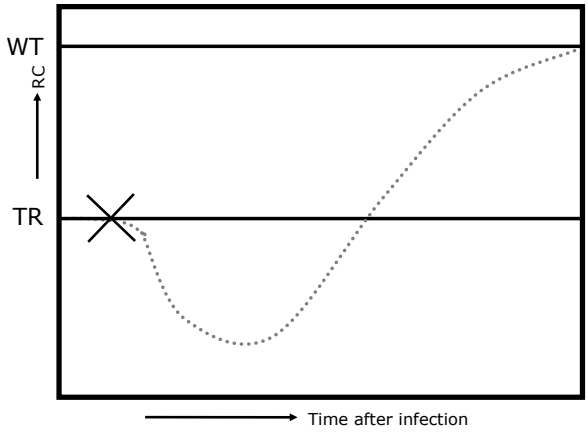


Figure 5. Reversion is prohibited by compensatory fixation. Due to compensatory mutations, multiple mutations are required for full reversion. The first mutation would decrease the RC, so reversion is prohibited.

mutations, as well as secondary mutations that may compensate at least partially for the loss of RC induced by the major mutations.

Moreover, we have preliminary data that suggest that compensatory fixation also explains persistence of a mutation in RT. The M41L mutation, which has a significant negative effect on replication capacity, persisted for up to 7 years in combination with potential compensatory mutations in a large cluster of untreated patients[53]. Similarly in our literature review the M41L mutation persisted in all but one patient.

Selection of additional compensatory mutations that offset the effects of transmitted drug-resistant variants on RC is another possibility leading to persistence due to compensatory fixation. We have shown *in vitro* that, in the absence of drug selection pressure PI-resistant viruses can select compensatory mutations which increase viral replication[52]. However, we did not find evidence for this evolutionary pathway in the reviewed literature; most likely because the papers focus on known primary drug-resistance mutations.

Implications of Transmitted Drug Resistance

As stated before, viruses with one or more drug resistance mutations generally have a reduced RC compared to wild-type virus in the absence of antiretroviral selection pressure. It has been suggested that due to the diminished RC, increases in viral load and reduction of $CD4^+$ cells may be slower in patients infected with drug-resistant HIV compared to patients infected with wild-type virus[54, 55]. Other groups did not confirm such observations[13, 56, 57].

Similarly, it may be expected that the disappearance of resistance mutations from the dominant replicating plasma population will increase the overall RC and viral fitness. Several reports indeed mention that replacement of T215Y or T215F by another variant resulted in a significant increase in HIV-1 RNA plasma levels[20, 22]. Disappearance of other drug resistance mutations has also been reported to coincide with increase of HIV RNA plasma levels[21, 23, 35]. A decline in $CD4^+$ levels after reversion has also been reported, suggesting that wild-type virus has a stronger negative effect on the $CD4^+$ turnover[23].

Transmission of drug-resistant variants may also influence clinical outcome. In the EuroSIDA cohort virological failure was retrospectively analyzed in treatment-naïve individuals who were infected with drug-resistant or drug-susceptible HIV-1[58]. Only a small and insignificant difference was found in terms of viral suppression. Unfortunately, it is not clear whether treatment was guided by genotypic and/or phenotypic analysis for optimal drug selection. Similar results were described by Oette *et al*. after analyzing a selection of 269 patients in whom therapy was selected after baseline genotypic and phenotypic analysis[59]. They did not observe any significant increase in virological failure in individuals infected with drug-resistant virus over those infected with fully sensitive virus. However, the potential adverse consequences of an infection with drug-resistant HIV are obvious, in that the efficacy of future treatment will be compromised if therapy is not modified. It has been shown that transmission of drug-resistant HIV can result in a delayed viral suppression and/or accelerated virological failure as result of the use of suboptimal therapy not guided by baseline resistance analysis. Recently, a large collaborative project European collaboration of HIV observational cohorts (EuroCoord) and the European Collaborative HIV and Anti-HIV Drug Resistance Network (CHAIN), looked into the effect of transmitted drug resistance on virological treatment outcome. Over 10000 patients were included in this analysis. In this study, patients infected with a resistant virus who received an NNRTI-based regimen had a higher risk of virological failure compared to patients infected with wild-type virus, even when this regimen was adapted to the presence of resistance mutations. This effect was not observed for patients receiving a therapy containing boosted PIs, probably due to the higher genetic barrier of these regimens[60]. These studies describe pooled analyses of different resistance profiles related to outcome of various regimens. Currently the generalizability of these data is uncertain. For instance it is not yet known whether the frequently observed transmission of single TAMs, which confer resistance to early NRTIs such as zidovudine, has a major impact on treatment regimens with a backbone of new NRTIs.

Furthermore, infections with revertants or atypical variants may also be relevant. Although they in general are not associated with reduced drug susceptibility, variants at position 215 of RT may lead to an increased ability to select the

resistance mutations as the genetic barrier to resistance is lowered[33]. The influence of 215 variants on virological outcome was retrospectively investigated in the Italian Cohort Naïve Antiretrovirals (ICONA) cohort. An increase in virological failure was observed in seroconverters with a 215 variant at baseline after initiation of a thymidine analogue-containing regimen (47% versus 30% among those without a 215 variant)[34].

The presence of revertants and atypical variants may also be an indicator of transmission of a more extensive resistance profile, as illustrated by Van Laethem *et al*.[61] They reported a distinctive case in which baseline population gene sequencing identified one major resistance mutation and one atypical variant. Therapy was selected using a genotypic interpretation algorithm, but no response was seen. Retrospective analysis using more sensitive sequencing techniques revealed the presence of additional resistance mutations in minority variants. These mutations were rapidly selected resulting in virological failure[61]. This case illustrates that resistant profiles based on population sequencing might be the tip of the iceberg (Figure 6). The possibility of more extensive underlying resistance should always be considered when selecting therapy in cases with transmitted resistance.

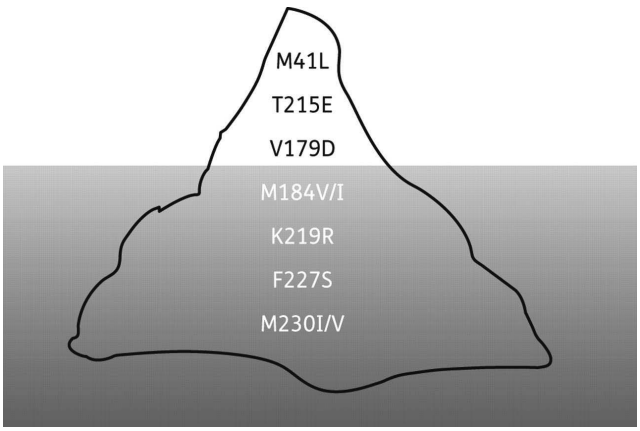


Figure 6. "Iceberg" model reflecting drug-resistance mutations in HIV-1 quasispecies. Only some of the mutations in the quasispecies can be detected above the water level, while minority variants harbouring additional mutations are below the surface and are not detected by population-based sequencing.

Comprehensive Detection of Transmitted Drug Resistance

In an effort to increase the detection of archived resistance mutations parallel analysis of DNA from peripheral blood mononuclear cell (PBMC) has been performed by several groups. In a study of 169 treatment naïve HIV-1 infected

individuals Vicenti *et al*. performed population sequence analysis on both plasma RNA and PBMC DNA[62]. Although the overall results did not show an increased sensitivity, in some cases additional drug-resistance mutations were found in PBMCs. Two groups have looked into transmitted resistance in both compartments. Comparing evolution in both compartments showed no differences in 3/5 cases, while in two patients 215Y and C mixtures were detected earlier in either plasma or PBMCs[27]. Another group only looked into PBMCs at the end of follow-up, revealing longer persistence of the RT mutation M184V in PBMCs[24]. Although these data are limited, it suggests that circulating PBMCs do not always serve as a representative archive for transmitted resistant HIV.

Additionally, clonal sequencing can also provide more insight in viral minorities present in the quasispecies. This type of analysis revealed gradual reversion towards wild-type for some drug resistance mutations[24, 28, 31].

New, even more sensitive techniques have provided more insight into the presence of resistant minority variants in plasma. Johnson *et al.* describe the use of a real-time PCR-based assay that is able to detect several minority drug resistance mutations (protease mutation L90M and RT mutations M41L, K70R, K103N, Y181C, M184V, and T215F/Y). In a cross-sectional analysis the sensitive PCR identified one or more minority drug resistance mutation(s) in 34/ 205 (17%) of newly diagnosed individuals who were first considered to be infected with drug sensitive HIV-1 based on conventional population sequencing. The clinical relevance of their observation was confirmed in a case-control study: 7/95 (7%) of those who experienced virological failure had minority drug resistance mutations at baseline compared with only 2/221 (0.9%) of treatment successes. Noteworthy, all patients were started on an NNRTI-based regimen. This data suggest that a considerable proportion of transmitted HIV-1 drug resistance goes undetected by conventional genotyping and that isolated minority mutations can have clinical consequences if a regimen with a low genetic barrier is initiated[63].

Discussion

Based on the available literature, we identified three different evolutionary pathways. Rapid evolution towards wild-type is observed for several drug-resistant variants with a profound effect on RC. Incomplete evolution towards wild-type may be observed if intermediates between the drug-resistant variant and wild-type are generated that have an RC that almost equals wild-type. A second pathway is evolution to atypical variants. These atypical variants do not represent intermediates, but equivalently result in a higher RC than the original transmitted resistant variant. Finally persistence of drug-resistant variants may be observed due to several underlying mechanisms. Mutations that induce only

limited decrease in RC often tend to persist. Furthermore, persistence of drug-resistant variants may also occur despite a reduced RC due to compensatory fixation. Such compensatory mutations may also be selected after transmission as additional mutations.

It is plausible that viral variants with a profound effect on replication capacity are transmitted less frequently[64] but no convincing evidence has been provided yet. This review shows that the relative under-representation in newly diagnosed patients of mutation M184V in RT, which is among the most common mutations in treated patients[65], can at least partially be explained by rapid reversion during follow-up according to this first evolution pathway[66].

It should be noted that there is considerable variability in the way the data were generated in the different studies. Several studies start the follow-up at the time of primary infection or directly after seroconversion while others determined the genotype later (2-16 weeks after seroconversion). The length of the follow-up after seroconversion varied from 2 to 58 months (median 18 months) and the interval between different genotypic analyses differs in each study. These variations make it difficult to make an estimate of the average time over which mutational patterns persist. In addition, several studies did not account for the presence or additional selection of secondary resistance mutations. They can significantly alter the RC of viruses harbouring primary resistance mutations and may therefore play a pivotal role in evolution after transmission. Despite these methodological variations and the limited availability of data, the general trends of evolution of mutations were very similar in all reviewed papers.

Selective pressure by the immune system may also influence evolution or persistence of transmitted resistance profiles. Since immune pressure is primarily exerted on the envelope and Gag and there is only a limited number of epitopes related to drug-resistant positions in PR and RT, the effect of the immune system on the viral evolution patterns covered in this review is most likely limited[67].

There is currently only limited information available on the clinical implications of transmitted drug resistance. Applying genotypic analysis prior to first-line therapy may detect drug resistance and may prevent virological failure. However, traditional genotyping even in combination with PBMC analysis does not always reveal the full pattern of transmitted resistance. Evidence is mounting that more extensive resistance can be detected using more sensitive techniques and the presence of major mutations as minority species at baseline can diminish treatment response, particularly to a regimen with a low genetic barrier[63, 68-70]. Individualized resistance testing and therapy, with the preferential use of regimens with a high genetic barrier may be of value in preventing failure of first-line therapy after infection with drug-resistant HIV-1.

In conclusion, different pathways of evolution of transmitted resistance patterns are observed. Data are being accumulated that specific transmitted drug-resistant viruses can persist and onward transmission of these variants to new hosts may occur. It is therefore important to continue monitoring appearance of drug-resistant viruses in populations on treatment and the rate of transmission in newly diagnosed patients.

References

1. Wawer MJ, Gray RH, Sewankambo NK et al. Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection, in Rakai, Uganda. *J Infect Dis* 2005; **191**: 1403-9.
2. Velasco-Hernandez JX, Gershengorn HB, Blower SM. Could widespread use of combination antiretroviral therapy eradicate HIV epidemics? *Lancet Infect Dis* 2002; **2**: 487-93.
3. Richman DD, Morton SC, Wrin T et al. The prevalence of antiretroviral drug resistance in the United States. *AIDS* 2004; **18**: 1393-401.
4. Van de Vijver DAMC, Wensing AMJ, Asjo B et al. Patterns of predicted drug susceptibility and its change over time among 2000 isolates across Europe: the CAPTURE study. *4th European HIV Drug Resistance Workshop*. Monaco, 2006.
5. Yerly S, Vora S, Rizzardi P et al. Acute HIV infection: impact on the spread of HIV and transmission of drug resistance. *AIDS* 2001; **15**: 2287-92.
6. Pao D, Fisher M, Hue S et al. Transmission of HIV-1 during primary infection: relationship to sexual risk and sexually transmitted infections. *AIDS* 2005; **19**: 85-90.
7. Brenner BG, Roger M, Routy JP et al. High rates of forward transmission events after acute/early HIV-1 infection. *J Infect Dis* 2007; **195**: 951-9.
8. Vercauteren J, Wensing AM, van de Vijver DA et al. Transmission of drug-resistant HIV-1 is stabilizing in Europe. *J Infect Dis* 2009; **200**: 1503-8.
9. Wensing AM, van de Vijver DA, Angarano G et al. Prevalence of drug-resistant HIV-1 variants in untreated individuals in Europe: implications for clinical management. *J Infect Dis* 2005; **192**: 958-66.
10. Weinstock HS, Zaidi I, Heneine W et al. The epidemiology of antiretroviral drug resistance among drug-naive HIV-1-infected persons in 10 US cities. *J Infect Dis* 2004; **189**: 2174-80.
11. Wheeler WH, Ziebell RA, Zabina H et al. Prevalence of transmitted drug resistance associated mutations and HIV-1 subtypes in new HIV-1 diagnoses, U.S.-2006. *AIDS* 2010; **24**: 1203-12.
12. Grant RM, Hecht FM, Warmerdam M et al. Time trends in primary HIV-1 drug resistance among recently infected persons. *JAMA* 2002; **288**: 181-8.
13. Little SJ, Holte S, Routy JP et al. Antiretroviral-drug resistance among patients recently infected with HIV. *N Engl J Med* 2002; **347**: 385-94.
14. Jain V, Liegler T, Vittinghoff E et al. Transmitted drug resistance in persons with acute/early HIV-1 in San Francisco, 2002-2009. *PLoS One* 2010; **5**: e15510.
15. Maglione M, Geotz M, Wang Z et al. Antiretroviral (ARV) Drug Resistance in the Developing World. Santa Monica, CA: Southern California Evidence-based Practice Center, 2007.
16. Barth RE, Wensing AM, Tempelman HA et al. Rapid accumulation of nonnucleoside reverse transcriptase inhibitor-associated resistance: evidence of transmitted resistance in rural South Africa. *AIDS* 2008; **22**: 2210-2.

17. Arrive E, Newell ML, Ekouevi DK et al. Prevalence of resistance to nevirapine in mothers and children after single-dose exposure to prevent vertical transmission of HIV-1: a meta-analysis. *Int J Epidemiol* 2007; **36**: 1009-21.
18. McIntyre JA, Hopley M, Moodley D et al. Efficacy of short-course AZT plus 3TC to reduce nevirapine resistance in the prevention of mother-to-child HIV transmission: a randomized clinical trial. *PLoS Med* 2009; **6**: e1000172.
19. Namukwaya Z, Mudiope P, Kekitiinwa A et al. The impact of maternal highly active antiretroviral therapy and short-course combination antiretrovirals for prevention of mother-to-child transmission on early infant infection rates at the Mulago national referral hospital in Kampala, Uganda, January 2007 to May 2009. *J Acquir Immune Defic Syndr* 2011; **56**: 69-75.
20. de Ronde A, van Dooren M, van Der Hoek L et al. Establishment of new transmissible and drug-sensitive human immunodeficiency virus type 1 wild types due to transmission of nucleoside analogue-resistant virus. *J Virol* 2001; **75**: 595-602.
21. Brenner BG, Routy JP, Petrella M et al. Persistence and fitness of multidrug-resistant human immunodeficiency virus type 1 acquired in primary infection. *J Virol* 2002; **76**: 1753-61.
22. Brenner B, Routy JP, Quan Y et al. Persistence of multidrug-resistant HIV-1 in primary infection leading to superinfection. *AIDS* 2004; **18**: 1653-60.
23. Chan KC, Galli RA, Montaner JS et al. Prolonged retention of drug resistance mutations and rapid disease progression in the absence of therapy after primary HIV infection. *AIDS* 2003; **17**: 1256-8.
24. Delaugerre C, Morand-Joubert L, Chaix ML et al. Persistence of multidrug-resistant HIV-1 without antiretroviral treatment 2 years after sexual transmission. *Antivir Ther* 2004; **9**: 415-21.
25. Barbour JD, Hecht FM, Wrin T et al. Persistence of primary drug resistance among recently HIV-1 infected adults. *AIDS* 2004; **18**: 1683-9.
26. Pao D, Andrady U, Clarke J et al. Long-term persistence of primary genotypic resistance after HIV-1 seroconversion. *J Acquir Immune Defic Syndr* 2004; **37**: 1570-3.
27. Ghosn J, Pellegrin I, Goujard C et al. HIV-1 resistant strains acquired at the time of primary infection massively fuel the cellular reservoir and persist for lengthy periods of time. *AIDS* 2006; **20**: 159-70.
28. Little SJ, Frost SD, Wong JK et al. Persistence of transmitted drug resistance among subjects with primary human immunodeficiency virus infection. *J Virol* 2008; **82**: 5510-8.
29. Polilli E, Di Masi F, Sozio F et al. Sequential transmission and long-term persistence of an HIV strain partially resistant to protease inhibitors. *New Microbiol* 2009; **32**: 205-8.
30. Yerly S, Rakik A, De Loes SK et al. Switch to unusual amino acids at codon 215 of the human immunodeficiency virus type 1 reverse transcriptase gene in seroconvertors infected with zidovudine-resistant variants. *J Virol* 1998; **72**: 3520-3.
31. Neifer S, Somogyi S, Schlote F et al. Persistence of a sexually transmitted highly resistant HIV-1: pol quasispecies evolution over 33 months in the absence of treatment. *AIDS* 2006; **20**: 2231-3.
32. Wensing AM, Van de Vijver D, Frentz D et al. Novel amino acids at resistance positions can indicate transmission of drug-resistant variants. *Antivir Ther* 2008; **2008**.
33. Garcia-Lerma JG, Nidtha S, Blumoff K et al. Increased ability for selection of zidovudine resistance in a distinct class of wild-type HIV-1 from drug-naive persons. *Proc Natl Acad Sci U S A* 2001; **98**: 13907-12.
34. Violin M, Cozzi-Lepri A, Velleca R et al. Risk of failure in patients with 215 HIV-1 revertants starting their first thymidine analog-containing highly active antiretroviral therapy. *AIDS* 2004; **18**: 227-35.

35. Gandhi RT, Wurcel A, Rosenberg ES et al. Progressive reversion of human immunodeficiency virus type 1 resistance mutations in vivo after transmission of a multiply drug-resistant virus. *Clin Infect Dis* 2003; **37**: 1693-8.
36. Johnson VA, Brun-Vezinet F, Clotet B et al. Update of the drug resistance mutations in HIV-1: December 2009. *Top HIV Med* 2009; **17**: 138-45.
37. Bennett DE, Camacho RJ, Otelea D et al. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. *PLoS One* 2009; **4**: e4724.
38. Nijhuis M, van Maarseveen NM, Lastere S et al. A novel substrate-based HIV-1 protease inhibitor drug resistance mechanism. *PLoS Med* 2007; **4**: e36.
39. Verheyen J, Knops E, Kupfer B et al. Prevalence of C-terminal gag cleavage site mutations in HIV from therapy-naive patients. *J Infect* 2009; **58**: 61-7.
40. Morozov VA, Morozov AV, Schurmann D et al. Transmembrane protein polymorphisms and resistance to T-20 (Enfuvirtide, Fuzeon) in HIV-1 infected therapy-naive seroconverters and AIDS patients under HAART-T-20 therapy. *Virus Genes* 2007; **35**: 167-74.
41. Peuchant O, Capdepont S, Ragnaud JM et al. Primary resistance to enfuvirtide (T20) in recently HIV-1 infected, antiretroviral-naive patients from the ANRS Aquitaine Cohort. *Antivir Ther* 2007; **12**: 559-62.
42. Young B, Fransen S, Greenberg K et al. Transmission of Integrase Strand-Transfer Inhibitor Multi-drug Resistant HIV: Case Report and Natural History of Response to Raltegravir-containing Antiretroviral Therapy. *Antivir Ther* 2011; **16(2):** 253-6.
43. Joos B, Fischer M, Kuster H et al. HIV rebounds from latently infected cells, rather than from continuing low-level replication. *Proc Natl Acad Sci U S A* 2008; **105**: 16725-30.
44. Keele BF, Giorgi EE, Salazar-Gonzalez JF et al. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A* 2008; **105**: 7552-7.
45. Martinez-Picado J, Martinez MA. HIV-1 reverse transcriptase inhibitor resistance mutations and fitness: a view from the clinic and ex vivo. *Virus Res* 2008; **134**: 104-23.
46. Nijhuis M, van Maarseveen NM, Boucher CA. HIV protease resistance and viral fitness. *Curr Opin HIV AIDS* 2007; **2**: 108-15.
47. Dykes C, Demeter LM. Clinical significance of human immunodeficiency virus type 1 replication fitness. *Clin Microbiol Rev* 2007; **20**: 550-78.
48. Back NK, Nijhuis M, Keulen W et al. Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. *EMBO J* 1996; **15**: 4040-9.
49. Cong ME, Heneine W, Garcia-Lerma JG. The fitness cost of mutations associated with human immunodeficiency virus type 1 drug resistance is modulated by mutational interactions. *J Virol* 2007; **81**: 3037-41.
50. Collins JA, Thompson MG, Paintsil E et al. Competitive fitness of nevirapine-resistant human immunodeficiency virus type 1 mutants. *J Virol* 2004; **78**: 603-11.
51. van Maarseveen NM, Wensing AM, de Jong D et al. Persistence of HIV-1 variants with multiple protease inhibitor (PI)-resistance mutations in the absence of PI therapy can be explained by compensatory fixation. *J Infect Dis* 2007; **195**: 399-409.
52. van Maarseveen NM, de Jong D, Boucher CA et al. An increase in viral replicative capacity drives the evolution of protease inhibitor-resistant human immunodeficiency virus type 1 in the absence of drugs. *J Acquir Immune Defic Syndr* 2006; **42**: 162-8.
53. Lindstrom A, Ohlis A, Huigen M et al. HIV-1 transmission cluster with M41L 'singleton' mutation and decreased transmission of resistance in newly diagnosed Swedish homosexual men. *Antivir Ther* 2006; **11**: 1031-9.

54. Harrison L, Castro H, Cane P et al. The effect of transmitted HIV-1 drug resistance on pre-therapy viral load. *AIDS* 2010; **24**: 1917-22.
55. Poggensee G, Kucherer C, Werning J et al. Impact of transmission of drug-resistant HIV on the course of infection and the treatment success. Data from the German HIV-1 Seroconverter Study. *HIV Med* 2007; **8**: 511-9.
56. Alteri C, Svicher V, Gori C et al. Characterization of the patterns of drug-resistance mutations in newly diagnosed HIV-1 infected patients naive to the antiretroviral drugs. *BMC Infect Dis* 2009; **9**: 111.
57. Bartmeyer B, Kuecherer C, Houareau C et al. Prevalence of transmitted drug resistance and impact of transmitted resistance on treatment success in the German HIV-1 Seroconverter Cohort. *PLoS One* 2010; **5**: e12718.
58. Bannister WP, Cozzi-Lepri A, Clotet B et al. Transmitted drug resistant HIV-1 and association with virologic and CD4 cell count response to combination antiretroviral therapy in the EuroSIDA Study. *J Acquir Immune Defic Syndr* 2008; **48**: 324-33.
59. Oette M, Kaiser R, Daumer M et al. Primary HIV drug resistance and efficacy of first-line antiretroviral therapy guided by resistance testing. *J Acquir Immune Defic Syndr* 2006; **41**: 573-81.
60. Wittkop LobotE-Cpt. Impact of transmitted drug resistance (TDR) on virological response to initial combination antiretroviral therapy (cART). *International HIV & Hepatitis Virus Drug Resistance Workshop & Curative Strategies* Dubrovnik, Croatia: Antiviral Therapy, 2010.
61. Van Laethem K, De Munter P, Schrooten Y et al. No response to first-line tenofovir+lamivudine+efavirenz despite optimization according to baseline resistance testing: impact of resistant minority variants on efficacy of low genetic barrier drugs. *J Clin Virol* 2007; **39**: 43-7.
62. Vicenti I, Razzolini F, Saladini F et al. Use of peripheral blood DNA for genotype antiretroviral resistance testing in drug-naive HIV-infected subjects. *Clin Infect Dis* 2007; **44**: 1657-61.
63. Johnson JA, Li JF, Wei X et al. Minority HIV-1 drug resistance mutations are present in antiretroviral treatment-naive populations and associate with reduced treatment efficacy. *PLoS Med* 2008; **5**: e158.
64. de Mendoza C, Rodriguez C, Corral A et al. Evidence for differences in the sexual transmission efficiency of HIV strains with distinct drug resistance genotypes. *Clin Infect Dis* 2004; **39**: 1231-8.
65. Yerly S, Jost S, Telenti A et al. Infrequent transmission of HIV-1 drug-resistant variants. *Antivir Ther* 2004; **9**: 375-84.
66. Toni TA, Asahchop EL, Moisi D et al. Detection of human immunodeficiency virus (HIV) type 1 M184V and K103N minority variants in patients with primary HIV infection. *Antimicrob Agents Chemother* 2009; **53**: 1670-2.
67. McMichael AJ, Rowland-Jones SL. Cellular immune responses to HIV. *Nature* 2001; **410**: 980-7.
68. Simen BB, Simons JF, Hullsiek KH et al. Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. *J Infect Dis* 2009; **199**: 693-701.
69. Halvas EK, Wiegand A, Boltz VF et al. Low frequency nonnucleoside reverse-transcriptase inhibitor-resistant variants contribute to failure of efavirenz-containing regimens in treatment- experienced patients. *J Infect Dis* 2010; **201**: 672-80.
70. Paredes R, Lalama CM, Ribaudo HJ et al. Pre-existing minority drug-resistant HIV-1 variants, adherence, and risk of antiretroviral treatment failure. *J Infect Dis* 2010; **201**: 662-71.

Chapter 4

Persistence of Transmitted Drug Resistant HIV-1 Variants explained by Compensatory Mutations

Marieke Pingen[1,2], Annemarie M.J. Wensing[2], Katrien Fransen[3], Annelies de Bel[4], Andy I.M. Hoepelman[5], Emmanouil Magiorkinis[6], Dimitrios Paraskevis[6], Maja M. Lunar[7], Mario Poljak[7], M. Nijhuis[2], Charles A.B. Boucher[1]

[1] Department of Virology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands;
[2] Virology, Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, the Netherlands;
[3] Institute of Tropical Medicine, Antwerpen, Belgium;
[4] AIDS Reference Laboratory of the Vrije Universiteit Brussel, subunit Universitair Ziekenhuis Brussels, Belgium;
[5] Internal Medicine and Infectious Diseases, University Medical Center Utrecht, Utrecht, the Netherlands;
[6] National Retrovirus Reference Center, Department of Hygiene, Epidemiology and Medical Statistics, Athens, Greece;
[7] University of Ljubljana, Ljubljana, Slovenia.

Manuscript in preparation

Abstract

In approximately 10% of newly diagnosed individuals in Europe , HIV variants carrying transmitted drug resistance mutations (TDRM) can be found. The majority of transmitted drug resistant HIV variants carry a single TDRM and resistance is limited to a single drug class. For some TDRM it has been shown they revert to wild type with a higher replicative capacity. However, other mutations tend to persist. To understand this persistence we investigated the *in vivo* evolution of frequently transmitted HIV variants and their effect on *in vitro* replicative capacity.

We selected 33 infected individuals with HIV harboring frequently transmitted resistance mutations and studied molecular evolution of protease and reverse transcriptase (RT) *in vivo.* The majority of resistance-related mutations (53/57) persisted for at least a year (10-14 months) and some even up to eight years. The most frequently encountered TDRM were introduced in a reference strain to investigate their impact on replicative capacity. Despite their observed persistence *in vivo* most mutations (M46I/M46L in protease and M41L, M41L+T215Y and K103N in RT) decreased viral replicative capacity, with only one exception, protease mutation L90M did not hamper viral replication. At baseline various polymorphisms at non-resistance related positions were present in all sequences (median number of polymorphisms protease: 6, RT: 8). During follow-up only limited selection of additional mutations was observed (median 1 additional polymorphism).

We propose that *in vivo* persistence of TDRM despite the reduction they confer to RC indicates that baseline polymorphisms functioned as compensatory mutations (partially) restoring the replicative capacity and constraining reversion of these TDRM.

Introduction

The viral enzymes reverse transcriptase (RT) and protease were the targets of the first antiretroviral drugs and the most commonly used therapy regimens still aim at inhibiting these viral proteins[1]. However, in resource-rich settings, in 10-15% of newly diagnosed patients HIV variants harboring drug resistance mutations in protease and RT are detected[2, 3]. The majority of transmitted viruses contain limited resistance profiles against single drug classes[2, 4]. Nucleoside RT inhibitors (NRTI) are the most frequently observed transmitted drug resistance mutations (TDRM). Especially thymidine analogue mutations M41L and T215 variants, that have been selected by drugs extensively used in the past, are often observed in newly diagnosed patients. A worrying trend is the increased prevalence of non-nucleoside RT inhibitor (NNRTI) related mutations in newly diagnosed patients, as single NNRTI mutations such as the frequently observed K103N result in high levels of resistance against multiple NNRTIs. In protease, M46I/L and L90M are the most frequently observed mutations[2, 3]. When present in combination with other protease mutations, both M46I/L and L90M are related to reduced susceptibility to several protease inhibitors (PIs)[5].

It is generally acknowledged that primary drug resistance mutations decrease the replicative capacity (RC) of HIV[6, 7] and as such in the absence of drugs can rapidly revert to wild type thereby increasing its RC. Indeed, follow-up of untreated individuals diagnosed with a drug resistant HIV variant revealed that certain mutations such as M184V in reverse transcriptase often revert after transmission to a new host[8, 9]. Increased detection of variants containing drug resistance mutations if very sensitive methods are applied confirms that reversion occurs frequently[10, 11]. However, some TDRM are known to persist for long periods, which may be explained by several mechanisms[8]. First, when the RC of a drug resistant variant approaches the RC of wild type virus, wild type has a limited replicative advantage, resulting in slow replacement of the resistant mutant. Alternatively, mutations that compensate for the decreased RC caused by the drug resistance mutation may have been selected in a previous or the current host. In this case reversion of either the resistance mutation or the compensatory mutation results in a decreased RC, all possible intermediates towards wild type will result in a "fitness valley". Therefore, reversion is prohibited. We proposed the term compensatory fixation for this phenomenon[8, 12, 13]. Finally, as several epitopes encompass resistance mutations escape from immune pressure could lead to persistence of TDRM[14, 15].

The majority of previous studies on evolution of transmitted drug resistant HIV variants were limited to small numbers of patients, often diagnosed with HIV variants harboring extensive resistance profiles[16-27]. Only one study investigated a larger number of recently infected patients[9]. However, these earlier studies

focused exclusively on the role and evolution of resistance mutations. The mechanisms underlying persistence of TDRMs still remain to be elucidated. To gain more insight in evolution of drug resistant HIV variants after transmission, we performed an extensive sequence analysis focusing on the most frequently occurring TDRM. Furthermore, we investigated the effect of the most frequently transmitted resistance mutations on replication efficacy.

Materials and Methods

In vivo evolution

Patients. Patients from four SPREAD-participating countries (Belgium, Greece, the Netherlands, Slovenia) were included. For all included patients, a baseline genotypic resistance test performed on a plasma sample within three months after diagnosis of HIV infection revealed at least one mutation on a position associated with transmitted drug resistance as described in the mutation list as recommended by the WHO[28]. The included patients were selected based on the observed TDRM profile at baseline. Follow-up genotypic analyses were performed one year (10-14 months) later and if available, at a third time point. All included patients were at least 18 years of age and not exposed to antiretroviral therapy during follow-up.

Sequence analysis. Genotypic resistance tests were performed by population sequencing of the viral protease and part of reverse transcriptase using commercially available assays or in-house methods covering at least amino acids 4-99 of protease and amino acids 30-249 of RT. All laboratories collaborated in a quality control program of ESAR to ensure high quality genotypic data[3, 4]. HIV subtype was determined using REGA 2.0[29]. To investigate evolution, the p-distance and the ratio of the proportions of synonymous and nonsynonymous substitutions (dS/dN ratio) were calculated using MEGA 5.05. The p-distance is the proportion of nucleotides between two sequences that has been changed. The dS/dN ratio, a measure of selection pressure[30], was calculated with the Nei-Gojobori method and statistically tested with a Z-test.

In vitro determination of replicative capacity

Virus panel. Mutations M46I, M46L and L90M in protease and M41L, M41L+T215Y and K103N in RT were introduced in HXB2 by site-directed mutagenesis using the previously described vector systems CP-MUT and NRT-MUT[31] and the following primers: M46I 5'-GGA AAC CAA AAA TAA TAG GG-3' (HXB2 nucleotides 2380-2396), M46L 5'-GGA AAC CAA AAC TGA TAG GG-3' (HXB2 nucleotides 2380-2396), L90M 5'-GAA ATC TGA TGA CTC AGA TTG-3' (HXB2 nucleotides 2511-2532), M41L 5'-ATT TGT ACA GAG CTG GAA AAG GAA G-3' (HXB2 nucleotides 2658-2682), K103N 5'-GTT ACT GAT TTG TTC TTT TTT AAC CC-3' (HXB2 nucleotides 2844-2869), T215Y 5'-TGT CTG GTG TGT

AAA GTC CCC ACC-3' (HXB2 nucleotides 3181-3204). Clones were verified by sequence analysis. Subsequently, recombinant virus stocks were generated by Lipofectamine 2000 (Invitrogen) transfection of HEK293T cells according to manufacturer's guidelines. Virus stocks were quantified by p24 analysis by ELISA (Aalto Bioreagent, Dublin, Ireland).

RC Analysis. Peripheral blood mononuclear cells (PBMCs) were prepared from healthy blood donors by Ficoll-Paque density gradient centrifugation. The replicative capacity of the virus panel was determined by infecting $5x10^6$ PHA-stimulated (2 mg/L) PBMCs with the equivalent of 40 ng p24 for two hours. Subsequently, cells were washed twice and maintained for 14 days in RPMI1640 with L-glutamine (BioWhittaker), 10% fetal bovine serum (Biochrom AG), 10 mg/L gentamicin (Gibco) and 5 U/ml IL-2. Cell-free supernatant was harvested daily for monitoring of the p24 production.

Results

Patients diagnosed with a transmitted drug resistant HIV variant

To investigate the evolution of transmitted drug resistant HIV variants, we selected 33 patients in four European countries (Belgium, Greece, the Netherlands, Slovenia) who were diagnosed in 2001 to 2008 with an HIV variant harboring a frequently observed resistance profile. The majority of these patients were men having sex with men (MSM), which is the most important route of transmission in Europe. Epidemiological studies demonstrated that most transmitted drug resistant HIV variants harbour resistance against a single drug class[3, 4]. In line with this previous observation, only 3/33 of the selected patients diagnosed with an HIV variant resistant to multiple drug classes were included in this study. A total of 57 resistance-associated mutations were observed in the transmitted viruses at baseline (Table 1). The median plasma HIV-RNA in our group of patients was 4.6 log copies/ml, slightly lower than the median HIV-RNA observed in the SPREAD cohort (4.8 log copies/ml). The baseline CD4 count was 617 cells/mm^3, which is higher than the median observed in SPREAD (343 cells/mm^3)[3]. This lower HIV-RNA and high CD4 count may either indicate that the selected viruses are less pathogenic, or that the included patients are more recently diagnosed. Prior negative HIV tests were available for 14 patients, revealing that at least nine patients had been infected for less than two years.

In vivo evolution of transmitted drug resistant HIV variants

For all patients, a second genotypic analysis one year after the baseline was performed. This revealed that the vast majority (53/57) of TDRM persisted during the first year of follow-up. Interestingly, in one of the patients diagnosed with a multi-drug resistant variant an additional resistance mutation (F53LF)

Table 1. Patient characteristics, resistance mutations and evolution.

ID	Gender	Last negative HIV test	Country of origin	Diagnosis	Risk group	Months after first analysis	Plasma HIV RNA (copies/ ml)	CD4 count	Sub -type	Resistance Profile PR	Resistance Profile RT	p-distance	p-value dN/dS
Transmitted variants harboring only NRTI-related mutations													
P01	male		NL	May 2007	MSM	0	>750000	461	B		M41L		
						10					M41L	0.001	1.000
						16					M41L	0.002	0.290
P02	male		NL	Jan 2008	BI	0	21800	423	B		M41L		
						12					M41L	0.005	0.225
						28					M41L	0.005	0.225
P03	male	Jan 2004	BE	Jun 2005	MSM	0	41000	483	B		M41L		
						11					M41L	0.000	1.000
						32					M41L	0.002	0.152
P04	female		NL	Mar 2001	HSX	0	6130	903	A		T69TP		
						10					-	0.000	1.000
						104					-	0.001	1.000
P05	male		NL	Feb 2007	MSM	0	102000	322	B		L210LS		
						11					-	0.000	1.000
						25					-	0.002	1.000
P06	male		SI	Jun 2001	MSM	0	12267	950	B		T215D		
						12					T215D	0.011	0.060
						99					T215D	0.007	0.428

Table 1. Continued

ID	Gender	Last negative HIV test	Country of origin	Diagnosis	Risk group	Months after first analysis	Plasma HIV RNA (copies/ml)	CD4 count	Sub -type	Resistance Profile PR	Resistance Profile RT	p-distance	p-value dN/dS
P07	male	Mar 2005	SI	Feb 2006	MSM	0	797000	953	B		T215S		
						14					T215S	0.000	1.000
						21					T215S	0.000	1.000
P08	male		NL	Sep 2008	MSM	0	36300	521	B		T215D		
						11					T215D	0.000	1.000
						27					T215D	0.000	1.000
P09	male	Sep 2004	NL	Dec 2004	MSM	0	583000	596	B		T215IT		
						13					-	0.000	
P10	male	Sep 2006	NL	Sep 2007	MSM	0	158000	678	B		T215AT		1.000
						13					T215AT	0.001	
						20					T215A	0.000	0.294
P11	male	Oct 2003	NL	Jan 2005	MSM	0	89800	289	B		K219N		1.000
						11					K219N	0.000	
						44					K219N	0.000	1.000
P12	male		BE	Mar 2006	HSX	0	318000	966	B		D67N T215C		
						13					D67N T215C	0.001	0.291
P13	male		NL	Feb 2007	MSM	0	55900	609	B		D67G T215C K219E		
						11					D67G T215C K219E	0.007	0.156
						24					D67G T215C K219E	0.000	1.000

Table 1. Continued

ID	Gender	Last negative HIV test	Country of origin	Diagnosis	Risk group	Months after first analysis	Plasma HIV RNA (copies/ ml)	CD4 count	Sub -type	Resistance Profile PR	Resistance Profile RT	p-distance	p-value dN/dS
P14	male	Jul 2004	NL	Nov 2007	MSM	0	294000	531	B		D67G T215C K219E		
						12					D67G T215C K219E	0.000	1.000
						14					D67G T215C K219E	0.000	1.000
P15	male	Apr 2005	NL	Jun 2005	HSX	0	750000	577	B		D67G T215C K219E		
						14					D67G T215C K219E	0.000	1.000
P16	male		NL	Aug 2005	MSM	0	81000	470	B		M41L T69S T210E T215ST		1.000
						11					M41L T69S T210DE T215ST	0.000	
						39					M41L T69S T210E T215ST	0.000	1.000
						77					M41L T69S T210E T215ST	0.000	1.000
P17	male	Mar 2005	NL	Jun 2006	MSM	0	34600	1129	B		M41L T69S T210E T215ST		
						13					M41L T69AS T210E T215ST	0.000	1.000
						33					M41L T69S T210E T215ST	0.001	1.000
						49					M41L T69S T210E T215ST	0.001	1.000

Table 1. Continued

ID	Gender	Last negative HIV test	Country of origin	Diagnosis	Risk group	Months after first analysis	Plasma HIV RNA (copies/ml)	CD4 count	Sub -type	Resistance Profile PR	Resistance Profile RT	p-distance	p-value dN/dS
Transmitted variants harboring only NNRTI-related mutations													
P18	male	Feb 2005	NL	Sep 2006	MSM	0	5990	790	B		K103N		
						12					K103N	0.000	1.000
						30					K103N	0.000	1.000
P19	male	Jun 2004	BE	Apr 2006	MSM	0	39900	648	B		K103N		
						12					K103N	0.000	1.000
						28					K103N	0.000	1.000
P20	male		NL	Sep 2005	MSM	0	21400	359	B		K103Q		
						12					K103Q	0.000	1.000
						59					K103Q	0.001	0.304
P21	male		NL	Jan 2004	HSX	0	2880	617	B		V179I		
						11					V179I	0.004	0.070
						59					V179I	0.000	1.000
P22	male	1995	SI	Sep 2005	MSM	0	29300	421	B		Y181C		
						11					Y181C	0.000	1.000
						49					Y181C	0.002	0.305
P23	Female		GR	Sep 2004	HSX	0	905	699	B		G190A		
						10			B		G190A	0.005	0.866
P24	male		GR	Jun 2004	?	0	10500	918	B		G190A		
						13			B		G190A	0.005	0.387

Table 1. Continued

ID	Gender	Last negative HIV test	Country of origin	Diagnosis	Risk group	Months after first analysis	Plasma HIV RNA (copies/ml)	CD4 count	Sub -type	Resistance Profile PR	Resistance Profile RT	p-distance	p-value dN/dS
Transmitted variants harboring only PI-related mutations													
P25	male		NL	Apr 2007	HSX	0	700000	664	B	M46L			
						14				M46L		0.000	1.000
						22				M46L		0.000	1.000
P26	male	Jan 2006	NL	Apr 2008	MSM	0	5170	742	B	M46L			
						10				M46L		0.001	0.310
						29				M46L		0.004	0.471
P27	male	Jul 2005	NL	Aug 2008	MSM	0	421000	409	B	M46L			
						14				M46L		0.000	1.000
						23				M46L		0.000	1.000
P28	male		NL	Aug 2008	MSM	0	111000	657	B	M46L			
						14				M46L		0.000	1.000
						26				M46L		0.001	0.299
P29	male	05-11-04	NL	Apr 2007	MSM	0	18100	699	B	M46L			
						13				M46L		0.000	1.000
						38				M46L		0.001	0.306
P30	male	15-02-03	NL	Mar 2005	MSM	0	69000	480	B	L90M			
						13				L90M		0.000	1.000

Table 1. Continued

ID	Gender	Last negative HIV test	Country of origin	Diagnosis	Risk group	Months after first analysis	Plasma HIV RNA (copies/ ml)	CD4 count	Sub -type	Resistance Profile PR	Resistance Profile RT	p-distance	p-value dN/dS
Transmitted variants harboring mutations against two drug classes													
P31	male		NL	Dec 2001	MSM	0	288	1468	B		D67G Y181CY T215C K219E		
						10					D67G T215C K219E	0.006	0.148
						46					D67G T215C K219E	0.000	1.000
P32	male		NL	Jan 2005	HSX	0	26600	667	B	G73S L90M	K103N		
						12				L90M	K103N	0.001	0.306
						18				L90M	K103N	0.001	0.306
P33	female		GR	Jul 2004	HSX	0	696	1288	B	I54V V82A L90M	M41L D67N L210W T215D		
						10				F53FL I54V V82A L90M	M41L D67N L210W T215D	0.001	0.293

Abbreviations: PR: protease; RT: reverse transcriptase; NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non- nucleoside reverse transcriptase inhibitor; PI: protease inhibitor; BE: Belgium; GR: Greece; NL: the Netherlands; SL: Slovenia; HSX: heterosexual; MSM: Men having sex with men; ?: unknown route of transmission

was detected as a mixture at one year after diagnosis. F53L may have been present at the detection limit of the genotypic assay, or was selected as additional mutation. For 28/33 patients a third genotypic analysis was performed at a median of 28 months (range: 14-99 months) after the first sample. During this extensive follow-up period, all resistance mutations persisted (Table 1).

Impact of frequently transmitted drug resistance variants on *in vitro* replicative capacity

Several papers have described the impact of some drug resistance mutations on the RC of HIV[32-37]. Many different methods have been used to investigate the RC, making data between studies incomparable. To our knowledge the impact of frequently transmitted protease and RT mutations was never compared. By introducing mutations M46I, M46L or L90M in protease or M41L, M41L+T215Y or K103N in RT in the background of HXB2, we determined the impact of frequently transmitted drug resistance mutations on the RC. As the wild type and drug resistant amino acid differ two nucleotides at position T215, many resistant, atypical and partial revertant variants are frequently observed. We selected the drug resistance amino acid T215Y as this TDRM is often observed in combination

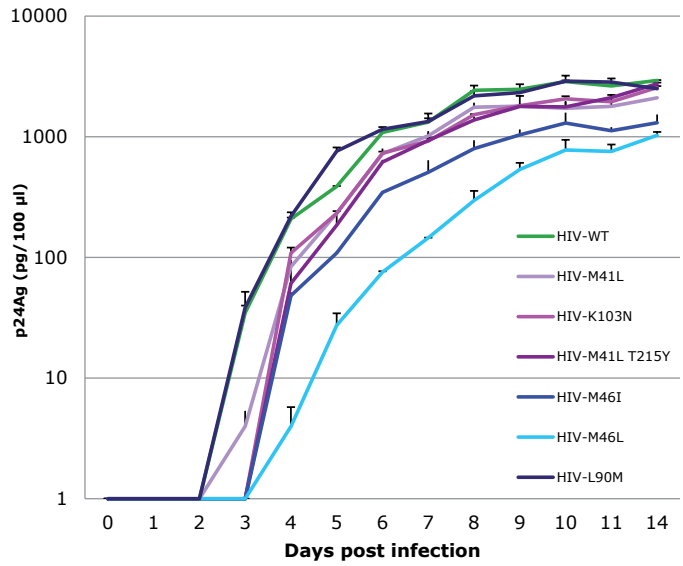


Figure 1. Impact of frequently observed transmitted drug-resistance mutations on viral replicative capacity. Replicative capacity of site-directed mutant viruses harboring frequently transmitted drug resistance mutations compared to HIV wild type. Equal amounts of virus were used to infect peripheral blood mononuclear cells in duplicate after which viral replication was monitored by p24 production. Error bars indicate the standard deviation.

with M41L[38]. Interestingly, except for mutation L90M in protease, all mutations caused a decrease in RC compared to HIV-WT. The reduction in RC of M41L, M41L+T215Y and K103N variants was quite comparable. M46I and M46L in protease resulted in the most severe reduction of RC.

Extensive analysis of *in vivo* evolution of transmitted drug resistant HIV variants

To investigate the potential selection of compensatory mutations increasing the replicative capacity in the current host, we analyzed the evolution of the transmitted viruses in more detail. The p-distance between baseline and follow-up sequences was calculated as a measure of evolution at the nucleotide level. For the majority of patients, this revealed a very low p-distance between baseline and one year, indicating limited evolution. In line with this observation, the dN/dS ratio of the viral populations, which is an indicator of selection, did not change significantly in any patient (Table 2). However, in all transmitted viruses changes in polymorphisms were observed which are described in Table 2.

Viruses harboring protease resistance mutations selected a median of 1 (range: 0-1) additional polymorphisms in protease during the first year of follow-up. Likewise, viruses harboring RT resistance mutations in RT selected a median of 1 (range 0-3) additional RT polymorphisms. Of course compensatory mutations may have been selected before diagnosis, or even in a previous host. Viruses harboring PR resistance mutations had a median of 6 (range: 4-9) additional polymorphisms at baseline. For RT, the median additional polymorphisms was 8 (range: 4-12).

Discussion

We investigated the evolution of transmitted drug resistant HIV variants in a group of patients diagnosed with frequently observed TDRM. The vast majority of TDRM persisted for at least a year up to eight years. Persistence of transmitted drug resistance mutations is in line with previous studies. Jain et al[9] demonstrated that except for M184V/I, the majority of TDR persist for longer than one year. The majority of patients in these studies had been diagnosed with a rare HIV variant with a complex resistance profile[8, 9]. In this study we focused on the single TDRM which are the most frequently transmitted variants as shown by large epidemiological studies[2, 4].

Determination of RC of site-directed mutant viruses revealed that the majority of frequently observed transmitted resistance mutations decrease the RC *in vitro* in PBMCs. Of the investigated mutant viruses, T215Y is known to evolve to atypical or partial revertant amino acids. Such alternative amino acids are known

Table 2. Evolution of transmitted drug resistant HIV variants.

ID	Months after first sample	Protease AA 4-99			Reverse transcriptase AA 41-230		
		Baseline	Reversion	Additional mutations	Baseline	Reversion	Additional mutations
Transmitted variants harboring only NRTI-related mutations							
P01	0	*S37N* L63P I93L			**M41L** V60I I135T S162C K166R R211G *L214F*		
	10					-K166R	+V106IV
	16						V106IV>I
P02	0	T12A K14KR Q18HQ L19IL *S37N* L63P I93L			**M41L** V60I I135T S162G K166EK I167F R211G *L214F*		
	12		-K14KR, -Q18HQ	L19IL>IKLQ		-S162G -I167F	K166EK>KR
	28		T12A>AT	L19IKLQ>IL		R211G>GR	+T165IT
P03	0	T12A I13V L19I *S37NS* I64V C67CR			**M41L** V60I F61FS *E122K* D123E I178L V179IV E203EG Q207EQ *L214F*		
	11		-S37NS -I64V>IV -C67CR	+I62IV		-F61FS -E203EG	+S162X V179IV>I Q207EQ>KQ +R211KR
	32		T12A>AT	I62IV>V		-S162X -I178L -V179I	Q207KQ>EQ

Table 2. Continued

ID	Months after first sample	Protease AA 4-99			Reverse transcriptase AA 41-230		
		Baseline	Reversion	Additional mutations	Baseline	Reversion	Additional mutations
P04	0	I13V K14R E35D M36I R41K R57K L63PS H69K L89M			K43R E44D **T69PT** D123NS K173S D177E V179I I202V Q207A R211S *L214F*		
	10			+*S37N*		-K43R>KR E44D>DE **-T69PT** R211S>RS	+Q197EQ
	104			+L10IL +G16EG +K70KR		-K43KR -E44DE -R211RS	D123NS>N +E169D K173S>LS Q197EQ>E
P05	0	E35D *S37D* D60E I62V L63P A71T I72V I93L			K49R V60I V118I *E122K* D123DE I135R S162D **L210LS** R211G		
	11			+I72V>EV		-L210LS	D123DE>E S162D>S162X +T200IT +E204EK
	25			+T12AT +K14KR +V77IV		-S162X, R211G>GR -E204EK	+T165IT
P06	0	*S37N* I64V			K64R R83K I178L I202V *L214F* **T215D**		
	12			+M36I		-K64R	+S68N +E122K
	99			+I13IV +K14KR +K45KR		R83K>KR, -I202V	+A158AS +S162T

Table 2. Continued

ID	Months after first sample	Protease AA 4-99			Reverse transcriptase AA 41-230		
		Baseline	**Reversion**	**Additional mutations**	**Baseline**	**Reversion**	**Additional mutations**
P07	0	L10I K14EV *S37N* L63T E65EV I72T V77I I93L			E122K I142V D177E Q207E *L214F* **T215S**		
	14			K14EV>E E65EV>V		-E122K>EK	
	21						
P08	0	I15L L19V *S37N* R41K D60E L63P I72IV I93L			V60I S68G R83K V90I A98S *E122P* D123DEG I135L S162C D177E I202IV R211K *L214F* **T215D**		
	11			+M36IM		-202IV	D123DEG>DE
	27		-M36IM	L19V>IV			D123DE>DEG +T200IT
P09	0	L10I *S37N* R41K I62V L63S V77I I93L			V60I S68G *E122K* I135V S162NS T165IT Q174HQ G196E R211G *L214F* **T215IT**		
	13					-S162NS -T165IT -T215IT	Q174HQ>H

Table 2. Continued

ID	Months after first sample	Protease AA 4-99			Reverse transcriptase AA 41-230		
		Baseline	Reversion	Additional mutations	Baseline	Reversion	Additional mutations
P10	0	*S37N* I62V L63T I64L V77I			S68T *E122K* I135V T139A G196E Q197R *L214F* **T215AT**		
	13					-T139A	+H198HR
	20			+R57KR		-H198HR	S68T>AT +T139AT +**T215AT>A**
P11	0	I15V E35D *S37D* D60E L63P V77I I93L			S68K T69N A98S L100LV *E122K* D123E I135R N136NT Q145E S162C I178M E194D I195L G196E T200A I202V Q207K R211G *L214F* **K219N** H221Y K223Q		
	11			+R41K		-L100LV -N136NT	
	44			+K45KR +R57KR		-I135R	+K49KR
P12	0	S37H R41KR R57K Q61D			V60I **D67N** T69E V106I D121Y I135T S162C D177E G196E E203D Q207E R211KR *L214F* **T215C**		
	13					-V106I L214F>FL	+T200IT
P13	0	L10I T12S L19I L63T			V60I **D67G** S68G I135T I178M R211KR *L214F* **T215C K219E**		
	11		-L10I	L19I>T		-R211KR	+E122EK
	24			+L10I L19T>I +I62IV		-E122EK -I135T	+Q207LQR +R211KR

Table 2. Continued

ID	Months after first sample	Protease AA 4-99			Reverse transcriptase AA 41-230		
		Baseline	Reversion	Additional mutations	Baseline	Reversion	Additional mutations
P14	0	T12S L19I L63T I64IM			V60I **D67G** S68G A158S I178M *L214F* **T215C** **K219E**		
	12			I64IM>M			+E40Q
	14					-E40Q	
P15	0	T12S L19T L63T			V60I **D67G** S68G I135IT I178M *L214F* **T215C** **K219E**		
	14						+E122EK I135IT>T
P16	0	L19I E35D *S37N* R57KR L63P V77IV I93L			**M41L T69S** D86DE *E122K* S162C I178L E204DE Q207EKQ **L210E** R211K *L214F* **T215ST**		
	11		-R57KR	V77IV>I			Q207EKQ>KQR **L210E>DE**
	39		V77I>IV	*S37N>DN* +R57KR			+V60VI +I195IL Q207KQR>x L210DE>E R211K>KN
	77		‘-R57KR			S162C>CS	E204DE>DEKN R211KN>K

Table 2. Continued

ID	Months after first sample	Protease AA 4-99			Reverse transcriptase AA 41-230		
		Baseline	Reversion	Additional mutations	Baseline	Reversion	Additional mutations
P17	0	L19I E35D *S37NS* L63P V77IV I93L			**M41L T69S** D86E K104KR *E122K* S162C I178L E204DE Q207KQR **L210E** R211DEKN *L214F* **T215S**		
	13			I72IM		-K104KR	**T69S>AS** S162C>CW
	33		-I72IM	E35D>DEKN *S37NS>N* V77IV>I		-E204DE	**T69AS>S** S162CW>W +E194DE Q207KQR>R R211DEKN>D
	49			E35DEKN>DE		-E194DE	Q207R>QR R211D>DEKN
P18	1	E35D R41K L63P I93L			**K103N** *E122K* D123E R211K *L214F*		
	12						+K173KT +D177DN
	30			*+S37N*		-K173KT	+Q174HQ +Q207QR R211K>KQ

Table 2. Continued

ID	Months after first sample	Protease AA 4-99			Reverse transcriptase AA 41-230		
		Baseline	Reversion	Additional mutations	Baseline	Reversion	Additional mutations
Transmitted variants harboring only NNRTI-related mutations							
P19	0	L10IV I13IV I15IV L19IL I62V L63PS I64LV C67S V77I			K64R **K103N** *E122K* D123E K173EK Q174QR V179I T200A R211K *L214F*		
	12		-L19IL	L10IV>I L63PS>X I64LV>V		-K173EK -Q174QR	+D177DN
	28						+R72RS +Q174QR
P20	0	L10I I15V *S37T* R41K C67G G68E H69R			V60I **K103Q** *E122K* D123E I142V R211K *L214F*		
	10						
	12						
	59			G68E>D			+ T200IT
P21	0	I15IV R41K L63H K70T I72V V77I			V60IV V90IV V108IV *E122KNRS* D123EG T139AT I142V S162C D177E I178L **V179I** T200A Q207E R211K *L214F*		
	11			I15IV>V		-V90IV -V108IV	V60IV>I E122KNRS>K D123EG>E T139AT>A +R206GR
	59			+I64IV		-T139A -R206GR	V60I>IV +K43KR E122K>S

Table 2. Continued

ID	Months after first sample	Protease AA 4-99			Reverse transcriptase AA 41-230		
		Baseline	Reversion	Additional mutations	Baseline	Reversion	Additional mutations
P22	0	T12N K14R *S37N* R41KR I64V			*E122K* D123E I135T **Y181C** T200A I202V R211K *L214F*		
	11		S37N>NS				
	49		K14R>KR -R41KR	+E35D S37NS>N +L63HQ		E122K>EK I135T>IT	D123E>AE
P23	0	I13V *S37NT* L63P A71AG			I50N G51W P52A V60IV R83K A98AG K101H S105LS D177E V179I **G190A** R211K *L214F*		
	10		-A71AG	S37NT>NST		-I50N -G51Q -P52A -A98AG -S105LS	V60IV>I +E122K K173EK
P24	0	I13V M36T *S37N* L63P			S48Q R83KR K101H D123DE D177E V179I **G190A** *L214F* H235R		
	13			M36T>IMT		-S48Q -H235R	D123DE>DEKN +S162CS
P25	0	*S37N* **M46L** D60E I62V L63S I72V V77I I93L			K49R V60I V118I I135R E169D R211G F214L		
	14						+F87FL +E204EK
	22					V60I>IV -F87FL -E204EK	

Table 2. Continued

ID	Months after first sample	Protease AA 4-99			Reverse transcriptase AA 41-230		
		Baseline	Reversion	Additional mutations	Baseline	Reversion	Additional mutations
P26	0	E35DE *S37N* **M46L** D60E I62V L63S I93L			K49KR V60I V118I *E122K* I135R R211G		
	10		-E35DE -I93L	+K70KR		-K49KR	
	29		-K70KR				+K104KR +S162C
P25	0	E35D *S37N* **M46L** D60E I62V L63S I93L			K49R V60I V118I *E122K* I135R R211G		
	14			+R41KR			+D123E
	23			L63S>PS I93L>IL			D123E>DEKN +I178ILV
Transmitted variants harboring only PI-related mutations							
P28	0	E35D *S37N* **M46L** D60E I62V L63S I93L			K49R V60I V118I *E122K* D123DN I135R R211G		
	14			+L19IL		-D123DN	+T165IT
	26			L19IL>X +A71AV			T165IT>I +E204EK
P29	0	E35D *S37N* **M46L** D60E I62V L63S I93L			K49R V60I V118I *E122K* I135R N136NT S162NS I167IT R211G		
	13			L63S>APS		-N136NT -S162NS -I167IT	
	38		-E35D	L63APS>A			

Table 2. Continued

ID	Months after first sample	Protease AA 4-99			Reverse transcriptase AA 41-230		
		Baseline	**Reversion**	**Additional mutations**	**Baseline**	**Reversion**	**Additional mutations**
P30	0	L19T *S37N* L63P **L90M** I93L			*E122K* T200A *L214F* K220X		
	13		S37N>NS			-K220X	
Transmitted variants harboring mutations against two drug classes							
P31	0	T4IT T12S L19IV L63X			V60I **D67G** S68G K70KR I178M **Y181CY** *L214F* **T215C K219E**		
	10		-T4IT	+L10I L19IV>I L63X>T		-K70KR **-Y181CY**	+I135IT +E204EG +R211KR
	46		-L10I	T12S>PS +G16AG L19I>IV +M36IM L63T>PT +I64IV		-E204EG -R211KR	I135IT>T
P32	0	L10I I13V I15V I62V L63P **G73S** **L90M**			V60I A98S **K103N** D121Y D123E I135T R172KR *L214F*		
	12		-G73S			I135T>IT -R172KR	
	18			+S37NS			+K102KR
P33	0	L10I I15V K20R E35D M36I *S37N* **I54V** Q58E L63P A71V **V82A** **L90M**			**M41L** K43N V60I **D67N** *E122P* I135T E138A I142V **L210W** R211M *L214F* **T215D**		
	10			+F53FL		-L214F	+T139I +I178IV

Patient-derived sequences are compared to HXB2. **Bold** positions indicate positions related to drug resistance, *italics* indicate polymorphisms of HXB2 compared to consensus B. Abbreviations: AA: amino acids.

to confer limited impact on viral RC[8, 20, 39], which is in line with the observed persistence of revertant and atypical T215 variants in our study.

Interestingly, K103N had a clear impact on replication in our experiments although this NNRTI-related mutation has been described to have a low impact in several[34, 35, 37] but not all[33] previous studies. This discrepancy may be due to the use of different assays or differences in replication caused by polymorphisms in lab strains. Additional experiments studying the effects of the most frequently observed TDRM in the genetic background of clinical isolates is ongoing.

Lack of reversion of the TDRM could be explained by a relatively small viral population size resulting in limited evolution. Although viral evolution was limited, in all transmitted viral variants changes at polymorphic sites were observed. Furthermore, the median plasma HIV-RNA level of the included patients is only slightly lower than generally observed in SPREAD[3], indicating that replication could result in molecular evolution.

Certain resistance mutations such as M46I in protease have been described to decrease recognition of epitopes by certain HLA types[15]. As a result, also the immune system may affect viral evolution and persistence of TDRM. However, the majority of frequently observed TDRM do not impact or can even enhance recognition of epitopes[14, 15] and as such, it is unlikely that the immune system is the driving force behind persistence of TDRM.

Therefore, it is likely that the lack of reversion is due to the presence of compensatory mutations restoring RC or resulting in compensatory fixation[12, 13]. Compensatory mutations may have direct structural interactions with resistance mutations, but can also affect the enzyme activity through other mechanisms. It has been demonstrated that mutations in gag may help to compensate the reduced protease activity conferred by resistance mutations in the protease itself[40]. For RT mutation M41L it has been described that V60I and S162A can compensate the decreased RC. Indeed, in all patient viruses harboring M41L, V60I and/or mutations at position S162 were present at baseline. In addition, compensatory mutations may be present in the connection domain[41]. Determination of the RC of frequently transmitted patient-derived HIV variants and the impact of various polymorphisms on RC can provide more insights in the mechanisms causing persistence.

The majority of the included patients were diagnosed in recent calendar years, and for only 9/33 patients a negative HIV tests within two years before diagnosis was available. We cannot exclude that patients were initially infected with a viral variant harboring a more extensive resistance profile and that some of these mutations had reverted before the patients were diagnosed. As such,

the observed limited evolution of *pol* may be a result of viral adaptation before diagnosis or may even have taken place in previous hosts. Indeed, phylogenetic studies have demonstrated that onward transmission by untreated patients is a major source of HIV transmission[42, 43]. In addition, we have previously used ultra-deep sequencing to investigate the quasispecies in plasma of 10 patients who were newly diagnosed with an HIV variant harboring a single NRTI-related resistance mutation. In 9/10 patients we were unable to detect viral minority variants harboring more extensive resistance profiles, which may be suggestive of infection with a circulating HIV variant harboring a limited resistance profile[44]. It is not unlikely that onward transmission of highly stable HIV variants harboring limited resistance profiles greatly contributes to the current epidemic of transmitted drug resistant HIV variants.

It is of great clinical importance to be able to distinguish whether transmitted drug resistant HIV variants harbor complex but partially reverted resistance profiles or circulating HIV variants containing limited resistance profiles. For the frequently observed NNRTI-resistance mutation K103N, it is well-known that it causes high levels of resistance against all first generation NNRTIs[45, 46]. Even when K103N is present as minority variant, it can contribute to therapy failure[47]. Fortunately, the recently approved second-generation NNRTIs remain active against HIV harboring K103N[48, 49]. In contrast, we have demonstrated that the NRTI-related M41L in RT has limited impact on selection of resistance against currently used NRTIs[50]. There is limited knowledge of the impact of M46I/L and L90M in protease on commonly used protease inhibitors.

In conclusion, the most frequently transmitted resistance mutations decrease the viral RC. However, these same mutations tend to persist *in vivo* which is likely due to presence of compensatory mutations selected in the current or previous host. This stability of transmitted drug resistant HIV variants facilitates onward transmission of these viruses.

Acknowledgements and funding

We acknowledge the support of contributors to the SPREAD surveillance program and members of the European Society for Antiviral Resistance (ESAR). We thank Dorien de Jong and Leander Theissen for technical assistance and Daniel Struck from the ESAR-team in Luxembourg for assistance in data management. This work was supported by the Aids fonds grant 2007034.

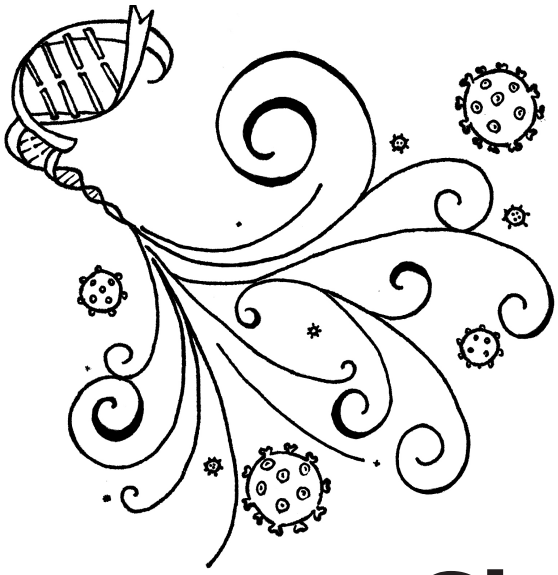
References

1. Arts EJ, Hazuda DJ. HIV-1 Antiretroviral Drug Therapy. *Cold Spring Harb Perspect Med* 2012; **2**: a007161.
2. Wheeler WH, Ziebell RA, Zabina H et al. Prevalence of transmitted drug resistance associated mutations and HIV-1 subtypes in new HIV-1 diagnoses, U.S.-2006. *AIDS* 2010; **24**: 1203-12.
3. Vercauteren J, Wensing AM, van de Vijver DA et al. Transmission of drug-resistant HIV-1 is stabilizing in Europe. *J Infect Dis* 2009; **200**: 1503-8.
4. Transmission of drug-resistant HIV-1 in Europe remains limited to single classes. *AIDS* 2008; **22**: 625-35.
5. Johnson VA, Calvez V, Gunthard HF et al. 2011 update of the drug resistance mutations in HIV-1. *Top Antivir Med* 2011; **19**: 156-64.
6. Martinez-Picado J, Martinez MA. HIV-1 reverse transcriptase inhibitor resistance mutations and fitness: a view from the clinic and ex vivo. *Virus Res* 2008; **134**: 104-23.
7. Nijhuis M, van Maarseveen NM, Boucher CA. Antiviral resistance and impact on viral replication capacity: evolution of viruses under antiviral pressure occurs in three phases. *Handb Exp Pharmacol* 2009: 299-320.
8. Pingen M, Nijhuis M, de Bruijn JA et al. Evolutionary pathways of transmitted drug-resistant HIV-1. *J Antimicrob Chemother* 2011; **66**: 1467-80.
9. Jain V, Sucupira MC, Bacchetti P et al. Differential persistence of transmitted HIV-1 drug resistance mutation classes. *J Infect Dis* 2011; **203**: 1174-81.
10. Li JZ, Paredes R, Ribaudo HJ et al. Low-frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: a systematic review and pooled analysis. *JAMA* 2011; **305**: 1327-35.
11. Wainberg MA, Moisi D, Oliveira M et al. Transmission dynamics of the M184V drug resistance mutation in primary HIV infection. *J Antimicrob Chemother* 2011; **66**: 2346-9.
12. van Maarseveen NM, Wensing AM, de Jong D et al. Persistence of HIV-1 variants with multiple protease inhibitor (PI)-resistance mutations in the absence of PI therapy can be explained by compensatory fixation. *J Infect Dis* 2007; **195**: 399-409.
13. Huigen MC, Albert J, Lindstrom A et al. Compensatory Fixation Explains Long Term Persistence of the M41L in HIV-1 Reverse Transcriptase in a Large Transmission Cluster. *15th International HIV Drug Resistance Workshop: Basic Principles and Clinical Implications*. Sitges, Spain: Antiviral Therapy, 2006; S113.
14. Mason RD, Bowmer MI, Howley CM et al. Antiretroviral drug resistance mutations sustain or enhance CTL recognition of common HIV-1 Pol epitopes. *J Immunol* 2004; **172**: 7212-9.
15. Mueller SM, Spriewald BM, Bergmann S et al. Influence of major HIV-1 protease inhibitor resistance mutations on CTL recognition. *J Acquir Immune Defic Syndr* 2011; **56**: 109-17.
16. Barbour JD, Hecht FM, Wrin T et al. Persistence of primary drug resistance among recently HIV-1 infected adults. *AIDS* 2004; **18**: 1683-9.
17. Brenner B, Routy JP, Quan Y et al. Persistence of multidrug-resistant HIV-1 in primary infection leading to superinfection. *AIDS* 2004; **18**: 1653-60.
18. Brenner BG, Routy JP, Petrella M et al. Persistence and fitness of multidrug-resistant human immunodeficiency virus type 1 acquired in primary infection. *J Virol* 2002; **76**: 1753-61.
19. Chan KC, Galli RA, Montaner JS et al. Prolonged retention of drug resistance mutations and rapid disease progression in the absence of therapy after primary HIV infection. *AIDS* 2003; **17**: 1256-8.

20. de Ronde A, van Dooren M, van Der Hoek L et al. Establishment of new transmissible and drug-sensitive human immunodeficiency virus type 1 wild types due to transmission of nucleoside analogue-resistant virus. *J Virol* 2001; **75**: 595-602.
21. Delaugerre C, Morand-Joubert L, Chaix ML et al. Persistence of multidrug-resistant HIV-1 without antiretroviral treatment 2 years after sexual transmission. *Antivir Ther* 2004; **9**: 415-21.
22. Ghosn J, Pellegrin I, Goujard C et al. HIV-1 resistant strains acquired at the time of primary infection massively fuel the cellular reservoir and persist for lengthy periods of time. *AIDS* 2006; **20**: 159-70.
23. Little SJ, Frost SD, Wong JK et al. Persistence of transmitted drug resistance among subjects with primary human immunodeficiency virus infection. *J Virol* 2008; **82**: 5510-8.
24. Neifer S, Somogyi S, Schlote F et al. Persistence of a sexually transmitted highly resistant HIV-1: pol quasispecies evolution over 33 months in the absence of treatment. *AIDS* 2006; **20**: 2231-3.
25. Pao D, Andrady U, Clarke J et al. Long-term persistence of primary genotypic resistance after HIV-1 seroconversion. *J Acquir Immune Defic Syndr* 2004; **37**: 1570-3.
26. Polilli E, Di Masi F, Sozio F et al. Sequential transmission and long-term persistence of an HIV strain partially resistant to protease inhibitors. *New Microbiol* 2009; **32**: 205-8.
27. Yerly S, Rakik A, De Loes SK et al. Switch to unusual amino acids at codon 215 of the human immunodeficiency virus type 1 reverse transcriptase gene in seroconvertors infected with zidovudine-resistant variants. *J Virol* 1998; **72**: 3520-3.
28. Bennett DE, Camacho RJ, Otelea D et al. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. *PLoS One* 2009; **4**: e4724.
29. de Oliveira T, Deforche K, Cassol S et al. An automated genotyping system for analysis of HIV-1 and other microbial sequences. *Bioinformatics* 2005; **21**: 3797-800.
30. Nei M, Gojobori T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol Biol Evol* 1986; **3**: 418-26.
31. van Maarseveen NM, Huigen MC, de Jong D et al. A novel real-time PCR assay to determine relative replication capacity for HIV-1 protease variants and/or reverse transcriptase variants. *J Virol Methods* 2006; **133**: 185-94.
32. Nicastri E, Sarmati L, d'Ettorre G et al. Replication capacity, biological phenotype, and drug resistance of HIV strains isolated from patients failing antiretroviral therapy. *J Med Virol* 2003; **69**: 1-6.
33. Cong ME, Heneine W, Garcia-Lerma JG. The fitness cost of mutations associated with human immunodeficiency virus type 1 drug resistance is modulated by mutational interactions. *J Virol* 2007; **81**: 3037-41.
34. Armstrong KL, Lee TH, Essex M. Replicative fitness costs of nonnucleoside reverse transcriptase inhibitor drug resistance mutations on HIV subtype C. *Antimicrob Agents Chemother* 2011; **55**: 2146-53.
35. Collins JA, Thompson MG, Paintsil E et al. Competitive fitness of nevirapine-resistant human immunodeficiency virus type 1 mutants. *J Virol* 2004; **78**: 603-11.
36. Imamichi T, Berg SC, Imamichi H et al. Relative replication fitness of a high-level 3'-azido-3'-deoxythymidine-resistant variant of human immunodeficiency virus type 1 possessing an amino acid deletion at codon 67 and a novel substitution (Thr-->Gly) at codon 69. *J Virol* 2000; **74**: 10958-64.
37. Xu HT, Oliveira M, Quan Y et al. Differential impact of the HIV-1 non-nucleoside reverse transcriptase inhibitor mutations K103N and M230L on viral replication and enzyme function. *J Antimicrob Chemother* 2010; **65**: 2291-9.
38. SPREAD. Recent dynamics of transmitted drug resistance in Europe: SPREAD programme 2006-2007. . *9th European Workshop on HIV & Hepatitis Treatment*

Strategies & Antiviral Drug Resistance. Paphos, Cyprus: Reviews in Antiviral Therapy & Infectious Diseases, 2011; 15-6.

39. Garcia-Lerma JG, Nidtha S, Blumoff K et al. Increased ability for selection of zidovudine resistance in a distinct class of wild-type HIV-1 from drug-naive persons. *Proc Natl Acad Sci U S A* 2001; **98**: 13907-12.
40. Clavel F, Mammano F. Role of Gag in HIV Resistance to Protease Inhibitors. *Viruses* 2010; **2**: 1411-26.
41. von Wyl V, Ehteshami M, Symons J et al. Epidemiological and biological evidence for a compensatory effect of connection domain mutation N348I on M184V in HIV-1 reverse transcriptase. *J Infect Dis* 2010; **201**: 1054-62.
42. Brenner BG, Roger M, Routy JP et al. High rates of forward transmission events after acute/early HIV-1 infection. *J Infect Dis* 2007; **195**: 951-9.
43. Yerly S, Vora S, Rizzardi P et al. Acute HIV infection: impact on the spread of HIV and transmission of drug resistance. *AIDS* 2001; **15**: 2287-92.
44. Pingen M, van der Ende M, Wensing A et al. Deep sequencing does not reveal additional transmitted mutations in patients diagnosed with HIV-1 variants with single nucleoside reverse transcriptase inhibitor resistance mutations. *HIV Med* 2012.
45. Bacheler L, Jeffrey S, Hanna G et al. Genotypic correlates of phenotypic resistance to efavirenz in virus isolates from patients failing nonnucleoside reverse transcriptase inhibitor therapy. *J Virol* 2001; **75**: 4999-5008.
46. Kuritzkes DR, Lalama CM, Ribaudo HJ et al. Preexisting resistance to nonnucleoside reverse-transcriptase inhibitors predicts virologic failure of an efavirenz-based regimen in treatment-naive HIV-1-infected subjects. *J Infect Dis* 2008; **197**: 867-70.
47. Li JZ, Paredes R, Ribaudo H et al. Relationship between Minority NNRTI resistance mutations, adherence, and the risk of virologic failure. *AIDS* 2011.
48. Anta L, Llibre JM, Poveda E et al. Rilpivirine Resistance Mutations in HIV Patients Failing Non-Nucleoside Reverse Transcriptase Inhibitor-Based Therapies. *AIDS* 2012.
49. Azijn H, Tirry I, Vingerhoets J et al. TMC278, a next-generation nonnucleoside reverse transcriptase inhibitor (NNRTI), active against wild-type and NNRTI-resistant HIV-1. *Antimicrob Agents Chemother* 2010; **54**: 718-27.
50. Pingen M, Nijhuis M, Boucher CAB et al. The frequently transmitted M41L mutation does not affect the in vitro selection of resistance against tenofovir and emtricitabine. *6th International Workshop on HIV Transmission*. Rome, Italy: Infectious Diseases & Antiviral Therapy, 2011; 20.



Chapter 5

Evolution and viral characteristics of a long-term circulating resistant HIV-1 strain in a cluster of treatment-naive patients

L. Marije Hofstra[1], Monique Nijhuis[1], Marieke Pingen[1], Tania Mudrikova[2], Annelies Riezebos-Brilman[3], Alberdina M. Simoons-Smit[4], Petra M. Van Ham[1], Wouter F.W. Bierman[5], Annemarie M.J. Wensing[1].

[1] University Medical Center Utrecht, Virology, Utrecht, The Netherlands.
[2] University Medical Center Utrecht, Internal Medicine and Infectious Diseases, Utrecht, The Netherlands.
[3] University Medical Center Groningen, Virology, Groningen, The Netherlands.
[4] Lucas Andreas Hospital, Medical Microbiology, Amsterdam, The Netherlands.
[5] University Medical Center Groningen, Internal Medicine, Groningen, The Netherlands.

Accepted for publication by Journal of Antimicrobial Chemotherapy

Abstract

Background Transmitted resistant HIV may revert to wild type in absence of drug-pressure due to reduced replication capacity (RC). We observed eight therapy-naïve patients infected with HIV harbouring 4 mutations on NRTI-resistance related positions: M41L-T69S-L210E-T215S. If partial reverted resistance patterns like these are detected at baseline, concerns for more extensive resistance in the quasispecies often directs selection of first-line antiretroviral therapy (cART) towards more complex regimens.

Method Genotypic resistance testing and phylogenetic analysis was performed using *pol* sequences of 400 therapy-naïve and 1322 patients with ≥ 1 NRTI-related mutation. *RT* genes were cloned into a reference strain and RC was investigated.

Result Phylogenetic analysis showed that all eight patients are part of a transmission cluster (bootstrap-value 92%). The patients are residing in three distinct geographical regions and were either MSM or heterosexually infected. Prior negative serology and analysis of base-ambiguity demonstrated circulation for at least 7 years. I*n vivo* evolution showed a mixture with wildtype (T215S/T) in only one untreated patient more than six years after diagnosis. The reverted resistance pattern did not confer a substantial reduction in RC compared to wild-type, explaining its persistence *in vivo* and long-term circulation in the population. Four patients started cART; three of them received quadruple cART. All patients showed good virologic and immunologic response.

Conclusion Long-term circulation transcending distinct regions and transmission groups suggests reversion occurred in previous hosts in the transmission chain. Identification of clusters using epidemiological data and active partner tracing may broaden therapeutic options in case of transmitted resistance.

Introduction

Despite prevention efforts and availability of combination antiretroviral therapy (cART), HIV incidence in industrialized countries has not decreased. Approximately ten percent of newly diagnosed patients are infected with a strain harbouring at least one drug resistance mutation[1-3]. Recently we showed that MSM are particularly at risk for contracting such a virus[2]. Reports on transmission networks in Switzerland and Canada show that transmission of resistance often occur in clusters[4, 5].

We observed eight therapy-naïve patients infected with a virus with four mutations on nucleoside reverse transcriptase inhibitor (NRTI) resistance related positions: M41L, T69S, L210E and T215S. Mutations at codons 41, 210 and 215 are thymidine analogue mutations (TAMs) and mutations at position 69 are also frequently observed in patients exposed to thymidine analogues. T215S is a known revertant of the resistance mutation T215Y/F and L210E is a potential revertant of resistance mutation L210W.

In general, drug-resistant strains confer reduced replication capacity (RC) compared to wild-type[6, 7]. After transmission, RC may be improved by (incomplete) reversion to wild-type in the absence of drug pressure in the newly infected host[8]. Therefore it is often assumed that transmitted drug resistance reflects direct infection from drug-experienced individuals.

When such a reverted pattern is detected in naïve patients, the possibility that reversion occurred in this particular patient is usually taken into account. Concerns for more extensive resistance in the quasispecies then results in the choice of more complex initial regimens with a higher genetic barrier, increased pill burden, more frequent toxicity and elevated costs. However, recent studies have shown that drug-resistant variants can persist even despite a low RC via various mechanisms in the absence of therapy[8, 9]. As a result, treatment-naïve individuals can be a source of onward transmission of drug resistance[5, 10], minimizing the risk of more extensive resistance in the quasispecies. We studied viral characteristics, phylogenetics and *in vivo* HIV evolution to gain insight into the characteristics of an infection with this partial reverted resistant strain.

Methods

Eight patients with this specific resistance profile (M41L-T69S-L210E-T215S) were identified in 2004-2010. All patients participate in the ATHENA observational cohort, which was approved by the national institutional review board. Clinical, virological and therapy-related data were collected. HIV-RNA genotypic resistance analysis of the *pol* gene was performed in routine clinical practice. Genotypic Sensitivity Scores (GSS) were determined as the sum of

scores for each drug in the regimen based on the predicted level of resistance (1: susceptible, ½: (potential) low-level or intermediate resistance, 0: high-level resistance) in the Stanford HIVdb-algorithm version 6.2.

Phylogenetic analysis

The *pol* sequences of the eight patients and baseline sequences of newly diagnosed treatment-naïve HIV-patients in our center in 2004-2010 (*n*=400) and subsequently all sequences performed in our center that showed 1 or more mutations on NRTI-resistance related position in 2000-2010 (*n*=1322) were aligned using ClustalW. A maximum likelihood phylogenetic tree was calculated with the Tamura-Nei model of evolution using bootstrap analysis with 1000 replicates (MEGA 5.0).

Estimation of duration of infection

Data on previous negative serological testing was collected to give definitive insight into the window of infection. In addition, base ambiguity of *pol* was used, which has proven to be a useful tool to estimate the duration of infection. The cut-off of 0.5% ambiguous nucleotides was chosen based on data from the Swiss cohort indicating that >0.5% ambiguity provides strong evidence against a recent infection event, one year prior to sampling[11].

RC and phenotypic drug susceptibility

Viral RNA was isolated from five plasma samples of patients 3, 4, 5 and 6 and five randomly selected patients infected with wild type. Nested PCR amplicons containing the N-terminal part of *reverse transcriptase* (RT) gene were generated and cloned into an HXB2 backbone and sequenced[12]. Recombinant viruses were generated by transfecting 293T-cells with these HIV-constructs using lipofectamine. RC was determined in peripheral blood mononuclear cells: cells were infected for 2 hours with 40 ng of p24, washed twice, resuspended and maintained for 14 days. Once daily 300 µL of cell-free virus supernatant was harvested for p24 analysis. Drug susceptibility was determined in a multicycle assay[13].

Sequencing of proviral DNA

DNA was isolated from 450 µL full blood[14]. PCR reactions were performed to amplify the N-terminal part of RT using the Expand High Fidelity PCR kit (Roche)[12]. Three positive amplification reactions were pooled and sequenced.

Results

Phylogenetic analysis showed that all eight patients with this particular resistance profile (M41L-T69S-L210E-T215S) are part of one transmission cluster (Figure 1, bootstrap value 92%). To ensure clustering was not based on the presence of resistance-associated mutations only, the phylogenetic analysis was repeated

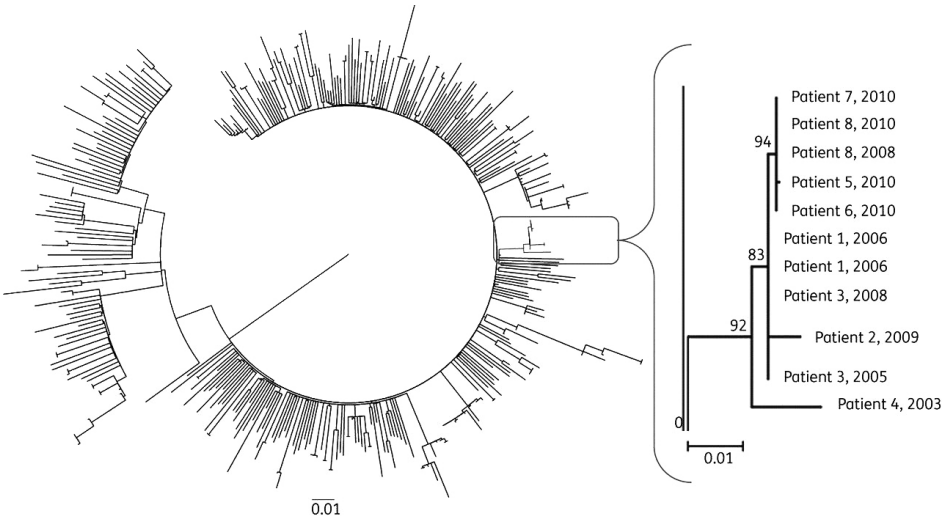


Figure 1. Phylogenetic tree. Maximum likelihood phylogenetic tree of therapy-naïve patients (2004-2010) (n=400). The box represents the transmission cluster of eight patients. The phylogeny of the cluster is shown in more detail with bootstrap values. Labels at tips of the trees correspond to the patient numbers in figure 2.

after manual reversion of the mutations to wild-type, resulting in the same cluster (bootstrap value 89%). Phylogenetic analysis including all sequences harbouring NRTI resistance mutations from different geographical regions did not reveal additional patients in the cluster nor a potential common source of the resistant strain.

The eight patients encompassing the transmission cluster were further characterized (Table 1). All patients are of Dutch ancestry and residing in three distinct regions in the country. Although the main route of transmission was MSM, two patients reported being infected by heterosexual contact. Patients 5 and 6 are known to be partners, as are patients 7 and 8. The CD4 counts at the time of HIV-1 diagnosis ranged from 180 to 1129 (median 670) cells/mm^3 and the plasma viral load (VL) ranged from 5000 to >100.000 (median 72800) copies/mL.

Data on the time of HIV diagnosis and prior negative testing allowed us to determine the period of circulation of this particular strain. The strain was first detected in 2003, when patient 4 tested positive for HIV. The other patients were diagnosed in the period 2005–10. We are certain, based on prior negative serological HIV testing in late 2008, that patient 7 was infected in 2009. Therefore we can conclude that the period of circulation is at least 6 years. Based on the low baseline CD4 count and the percentage of ambiguous nucleotides exceeding 0.5%, it is likely that patient 4 was chronically infected at the time of diagnosis

Table 1. Patient characteristics.

Pt	Sex	Age	Route of transmission	Date of diagnosis	Baseline CD4	Baseline VL	Start cART	Regimen	GSS
1	M	50	MSM	19-06-2006	1129	34600	October 2010	TDF/FTC, MVC, ATV/r	3.5
2	M	34	HSX	26-01-2009	512	5510			
3	M	52	MSM	25-08-2005	470	81000			
4	F	64	HSX	26-11-2003	180	55000	June 2004	ABC/3TC, NVP	2.5
5	M	43	MSM	09-09-2010	890	88100			
6	M	48	MSM	16-09-2010	690	135000			
7	M	43	MSM	14-10-2009	680	86800	October 2011	TDF/FTC, LPV/r, EFV	3.5
8	M	41	MSM	21-07-2008	660	64600	July 2010	TDF/FTC, LPV/r, EFV	3.5

MSM = homosexual, HSX = heterosexual, TDF/FTC = tenofovir/emtricitabine, ATV/r = ritonavir-boosted atazanavir, MVC = maraviroc, ABC/3TC= abacavir/lamivudine, NVP = nevirapine, LPV/r = ritonavir-boosted lopinavir, EFV = efavirenz, GSS = genotypic sensitivity score.

in 2003. Therefore we assume that this strain has been circulating in the Dutch population for at least 7 years (Figure 2).

In addition, we studied the *in vivo* evolution of the viral population in the four patients who did not initiate cART (follow up 18 – 77 months). The resistance pattern persisted in all but one patient, in which a mixture with wild-type (T215T/S) was observed more than 6 years after diagnosis. Sequences of proviral DNA at the time of diagnosis and after follow up (1.5 – 4 years after diagnosis) were available in two patients and no reversion was observed.

We hypothesized that the RC of this strain may play a role in persistence in treatment-naïve patients and circulation within the population. We compared the RC of the five patient-derived recombinant RT cluster clones to five randomly selected patient-derived wild-type RT strains and four reference strains (HXB2 and viral strains that harbour various mutations at codon 184 of RT). As expected, reference strain 184T is the least fit and HXB2 wild-type confers the highest RC. The five RT clones representing the cluster are all replicating as efficient as HXB2 wild-type, demonstrating that strains with this RT reversion pattern are not substantially reduced in RC (data not shown).

To date, four patients have received cART (Table 1). Three patients received quadruple cART, to ensure a GSS above 3. One patient received a combination of abacavir, lamivudine and nevirapine (GSS of 2.5). If calculation was based on

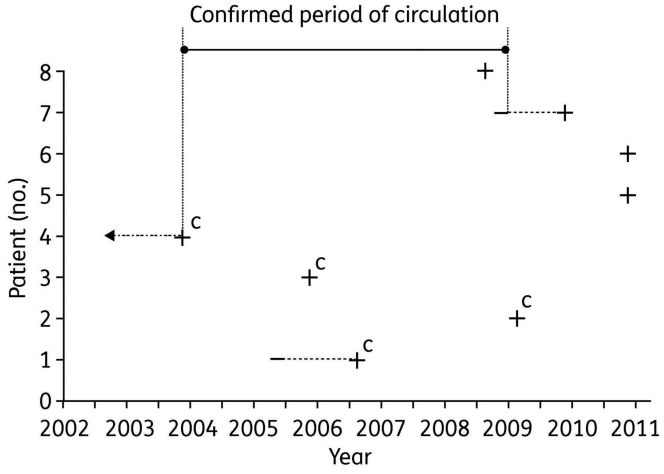


Figure 2. Epidemiological curve of time of diagnosis. + = first positive HIV test, - = last negative HIV test, c = base ambiguity > 0.5% suggesting no recent infection. The confirmed period of circulation is 6 years, based on date of diagnosis of patient 3 and the last negative HIV test of patient 7.

the most likely pre-reversion resistance pattern (M41L- T69S- L210W-T215Y/F), GSS dropped 0.5 point for all patients. However, all four patients achieved and maintained undetectable viral loads and all had a good immunologic response (mean CD4 count 593 cells/mm^3). Retrospective phenotypic drug susceptibility testing showed that the RT cluster-strain was susceptible for all tested NRTIs (lamivudine, zidovudine, abacavir and tenofovir) (data not shown).

Discussion

This study is the first to provide evidence of long-term circulation of a resistant HIV-1 strain among therapy-naïve patients based on prior negative serological testing. We showed that this variant has been circulating for more than 7 years within a cluster of therapy-naïve patients. Clusters with drug-resistant virus have been described before. Buskin *et al*.[15] observed the transmission of multiclass drug-resistant HIV within a cluster of MSM for >2-year. In 2009, Hue *et al*.[16] already showed the circulation of resistant lineages within untreated populations[16]. Based on assessment of the most recent common ancestor they estimated circulation for up to 8 years[16].

In contrast to acquired drug-resistant HIV-1 variants in treated patients, transmitted drug-resistant variants in treatment-naïve patients can persist for a long time in the absence of drug pressure[9]. This is surprising since the fitness costs of some drug resistance mutations present in our cluster-strain have been well-established[6]. However, we showed that the *RT* mutations in this cluster did not have an evident negative impact on the RC *in vitro*, which is in line with the slow reversion to wild-type *in vivo*.

Previous studies have shown that the persistence of drug-resistant mutations may be explained by selection of compensatory mutations that restore the RC of the virus[17]. In our cluster, we detected a previously described compensatory mutation, 60I, in the HIV-strain of one patient. It is possible that other mutations in this particular strain play a role as well.

The transmission clusters in the analysis of Hue *et al*.[16] included almost exclusively MSM and were restricted to one geographical area in the UK. We report circulation of drug resistance among therapy-naïve individuals transcending distinct regions throughout the country and different transmission groups. Our dataset covered only a subset of all newly diagnosed patients in the Netherlands. Taking into account the long-term circulation in different regions and risk groups, it is likely that transmission of drug resistance among therapy-naïve patients is even more extensive than we showed here. The existence of reservoirs of drug-resistance in therapy-naïve patients has important public

health implications and underlines the importance of baseline genotypic testing and the need for active testing policies to interrupt transmission chains.

Circulation of particular resistance patterns in therapy-naïve patients may also have consequences for clinical practice. Due to concerns for more extensive resistance in the quasispecies most clinicians initiated for the patients in our cluster complex quadruple cART for the patients in our cluster.

Although we cannot exclude the possibility of one drug-experienced source infecting all patients individually in the cluster, we could not identify such a source by phylogenetic analysis of the available sequences. However, the long-term circulation in different regions and risk groups makes the hypothesis of one source infecting all patients unlikely. We infer that reversion did not take place in these therapy-naïve patients but in a previous host earlier in the transmission chain. We showed that this particular strain was phenotypically susceptible to all NRTIs that are frequently used in clinical practice. Furthermore, the patient on triple therapy achieved and maintained a good virological and immunological response for more than 7 years. Therefore, we conclude that identification of clusters and active partner tracing may provide insight into the risk of more extensive resistance patterns in the quasispecies and prevent excessive use of antivirals.

Acknowledgements and funding

We thank Leander Theissen for his contribution to DNA sequencing. We also want to acknowledge the Utrecht Genoteam, especially Danielle van Heumen for performing genotypic analysis. This work was supported in part by the Aids Fonds (grant number 2007034).

References

1. Wensing, A. M., van de Vijver, D. A., Angarano, G. *et al.* Prevalence of drug-resistant HIV-1 variants in untreated individuals in Europe: implications for clinical management. *J Infect Dis* 2005; **192**: 958-966.
2. Frentz, D., van de Vijver, D. A., Abecasis, A. *et al.* Transmission of HIV resistant to non-nucleoside RT inhibitors is rising in MSM in Europe. In: *Abstract Book 13th European AIDS Conference, Belgrade, Serbia, 2011*. Abstract PS1/5, p.11. European AIDS Clinical Society, Brussels, Belgium.
3. Castor, D., Low, A., Evering, T. *et al.* Transmitted drug resistance and phylogenetic relationships among acute and early HIV-1-infected individuals in New York City. *J Acquir Immune Defic Syndr* 2012; **61**: 1-8.
4. Brenner, B. G., Roger, M., Moisi, D. D. *et al.* Transmission networks of drug resistance acquired in primary/early stage HIV infection. *Aids* 2008; **22**: 2509-2515.
5. Yerly, S., Junier, T., Gayet-Ageron, A. *et al.* The impact of transmission clusters on primary drug resistance in newly diagnosed HIV-1 infection. *Aids* 2009; **23**: 1415-1423.

6. Harrigan, P. R., Bloor, S. & Larder, B. A. Relative replicative fitness of zidovudine-resistant human immunodeficiency virus type 1 isolates in vitro. *J Virol* 1998; **72**: 3773-3778.
7. Deeks, S. G., Wrin, T., Liegler, T. *et al.* Virologic and immunologic consequences of discontinuing combination antiretroviral-drug therapy in HIV-infected patients with detectable viremia. *N Engl J Med* 2001; **344**: 472-480.
8. Pingen, M., Nijhuis, M., de Bruijn, J. A. *et al.* Evolutionary pathways of transmitted drug-resistant HIV-1. *J Antimicrob Chemother* 2011; **66**: 1467-1480.
9. Brenner, B. G., Routy, J. P., Petrella, M. *et al.* Persistence and fitness of multidrug-resistant human immunodeficiency virus type 1 acquired in primary infection. *J Virol* 2002; **76**: 1753-1761.
10. Audelin, A. M., Gerstoft, J., Obel, N. *et al.* Molecular phylogenetics of transmitted drug resistance in newly diagnosed HIV Type 1 individuals in Denmark: a nation-wide study. *AIDS Res Hum Retroviruses* 2011; **27**: 1283-1290.
11. Kouyos, R. D., von Wyl, V., Yerly, S. *et al.* Ambiguous nucleotide calls from population-based sequencing of HIV-1 are a marker for viral diversity and the age of infection. *Clin Infect Dis* 2011; **52**: 532-539.
12. Huigen, M. C., de Graaf, L., Eggink, D. *et al.* Evolution of a novel 5-amino-acid insertion in the beta3-beta4 loop of HIV-1 reverse transcriptase. *Virology* 2007; **364**: 395-406.
13. Boucher, C. A., Keulen, W., van Bommel, T. *et al.* Human immunodeficiency virus type 1 drug susceptibility determination by using recombinant viruses generated from patient sera tested in a cell-killing assay. *Antimicrob Agents Chemother* 1996; **40**: 2404-2409.
14. Boom, R., Sol, C. J., Salimans, M. M. *et al.* Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990; **28**: 495-503.
15. Buskin, S. E., Ellis, G. M., Pepper, G. G. *et al.* Transmission cluster of multiclass highly drug-resistant HIV-1 among 9 men who have sex with men in Seattle/King County, WA, 2005-2007. *J Acquir Immune Defic Syndr* 2008; **49**: 205-211.
16. Hue, S., Gifford, R. J., Dunn, D. *et al.* Demonstration of sustained drug-resistant human immunodeficiency virus type 1 lineages circulating among treatment-naive individuals. *J Virol* 2009; **83**: 2645-2654.
17. Lindstrom, A., Ohlis, A., Huigen, M. *et al.* HIV-1 transmission cluster with M41L 'singleton' mutation and decreased transmission of resistance in newly diagnosed Swedish homosexual men. *Antivir Ther* 2006; **11**: 1031-1039.

Chapter 6

Deep sequencing does not reveal additional transmitted mutations in patients diagnosed with HIV-1 variants with single NRTI resistance mutations

Marieke Pingen[1,2], Marchina E. van der Ende[3], Annemarie M. Wensing[2], Abdelilah el Barzouhi[3], Birgitte B. Simen[4], Martin Schutten[1] & Charles A. B. Boucher[1]

[1] Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands;
[2] Department of Virology, Medical Microbiology, University Medical Center Utrecht, Utrecht, the Netherlands;
[3] Department of Internal Medicine, Erasmus Medical Center, Rotterdam, the Netherlands;
[4] 454 Life sciences, a Roche company, Branford, CT, USA.

HIV Medicine, 2013; 14(3):176-81.

Abstract

Objective The aim of this study was to gain more insight into the relationship between transmitted singletons found at HIV diagnosis by population sequencing and the possible presence of clinically relevant viral minorities containing additional resistance mutations.

Methods We studied the viral quasispecies and therapy response in ten individuals with transmitted single NRTI-related resistance mutations as detected by population sequencing.

Results Ultra-Deep pyrosequencing did not reveal additional drug-resistant mutations in nine of ten patients. In these nine patients no breakthrough with resistant viruses was observed despite the use of low genetic NNRTI based regimens in the majority of patients.

Conclusion Viral minority variants containing additional resistance mutations are rare in patients with transmitted NRTI singletons in the Netherlands. Larger studies are required to confirm these findings and to determine the therapeutic consequences.

Introduction

Epidemiological studies in the developed world using population sequencing show that at least 10% of all newly diagnosed HIV-1 patients are infected with a variant containing drug-resistance mutations. Frequently, this involves NRTI-related resistance mutations[1, 2]. In the majority of patients newly diagnosed with transmitted drug resistance (TDR) in Europe, only a single mutation (singleton) is detected[3]. However, the population-based sequencing techniques used for epidemiologically studies and in clinical practice have a detection limit of 10-25% and thus can miss additional mutations when present as a minority. Newer, more sensitive methods revealed the presence of resistant minority variants undetected by population sequencing, indicating that the actual rate of transmission of drug resistance may be higher[4, 5]. This could be explained by reversion of drug resistance mutations in the absence of drugs[6]. As a consequence, transmitted drug resistance is no longer detectable by population sequencing of the plasma, but may persist as minority variant in plasma or as archived provirus[7, 8]. Such undetected resistant minority variants have been reported to increase the risk of therapy failure[9].

The objective of this study was to gain more insight into the relationship between singletons found at HIV diagnosis by population sequencing and the possible presence of clinically relevant viral minorities containing a more complex resistance profile. We performed ultra-deep pyrosequencing (UDPS) and investigated response to therapy in a group of men having sex with men (MSM) that were diagnosed in the Netherlands between 2003 and 2008.

Methods

Patients/samples

Population-based sequencing was used to identify transmitted drug resistance for all newly diagnosed men having sex with men in our center since 2003 according to the WHO list of mutations related to transmitted drug resistance[10]. The WHO list includes mutations selected under pressure of drugs but also some "intermediate" and "atypical" mutations. Intermediate codons can appear if the difference between the wild type and the resistance codon is two nucleotides. Replication of the resistant variant in the absence of drug may lead to the reversion of just one of the two nucleotides, leading to an intermediate amino acid at that position. Additionally, novel atypical amino acids that are not an intermediate towards the wild type can be selected. This is frequently observed at position 215 in Reverse Transcriptase (RT), where the difference between wild type thymidine (T) and resistance codons Tyrosine (Y) and Phenylalanine (F) is two nucleotides. We have included all atypical mutations at the resistance positions described in the WHO list.

Patients were selected for this study when they were diagnosed between 2003 and 2008 in our hospital, had a single nucleoside/tide reverse transcriptase inhibitors (NRTI) related mutation at baseline and had started therapy. Ten patients met these selection criteria. Patient characteristics are summarized in the Table. All included patients have signed a consent form in which they state that anonymized data and materials sent in for routine diagnostics could be used for research.

Population based sequence analysis

Population-based sequence analysis of *pol* was performed on the earliest available sample. Plasma samples were stored at -80ºC until time of genotypic analysis. RNA isolation and sequence analysis was performed according to the ViroSeq HIV-1 genotyping protocol (Abbott Molecular, Hoofddorp, the Netherlands).

UDPS

The earliest available sample was used to perform UDPS (454 Life Sciences, a Roche Company, Brandford, CT, USA). In eight cases, the same sample was also used for population-based sequencing. For two patients, a later sample was used for UDPS; 2 weeks later for patient B and 6 months later for patient C. RNA was extracted from 500 µl plasma using the ViroSeq isolation method. UDPS was performed as described by Simen et al.[11] Briefly, three overlapping cDNA fragments covering protease and the n-terminus of RT were amplified using Superscript II enzyme (Invitrogen, Breda, the Netherlands) and three primers. Subsequently, eight amplicons were generated by amplifying cDNA using the FastStartHiFi PCR system (Roche Applied Science, Almere, the Netherlands) and pyrosequencing was performed. Previous use of the same UDPS method showed that with RNA isolated from 140 µl plasma with a high HIV RNA concentration, minorities of ≤1% could repeatedly be detected. As 500 µl plasma with a high HIV-RNA was used, the lower detection limit for mutations was set at 1% in this study[11].

Clinical follow-up

Subsequent response to virological treatment was monitored for all patients.

Results

Patient characteristics

All patients were MSM and were diagnosed between 2003 and 2008. Their median age was 37 years old (range: 25-54 years). Two patients had a known negative HIV-1 test within one year before diagnosis, two within 2 years before diagnosis. Overall, from seven patients a negative HIV-1 test was obtained in five years preceding their diagnosis (See Table 1).

Median CD4 count at baseline was 240 (range: 30-490), and all patients had an HIV-1 RNA plasma level above the upper limit of quantification of 100,000 copies/ml

and were therapy-naive at the time of the baseline genotypic analysis. Population based sequence analysis of the *pol* gene on a sample at time of diagnosis (max 1.5 months later) showed single resistance mutations in all patients (M41L, T69N, M184V, T215L, K219Q, T215E, T215S). The average time between diagnosis and therapy initiation was 9 months (range: 1-49 months). Patient characteristics, results of population-based genotypic analysis and therapy are described in Table 1.

UDPS

All samples had a high HIV RNA level enabling a high number of successful reads and thus a good representation of the viral population in plasma. This resulted in successful forward and reverse reads with a range of 1659-3140 reads per site (Median: 2558). A comparison of the population-based genotypic analysis and UDPS is shown in Table 1.

The results of the UDPS were very similar to the population-based genotypic analysis. For 9/10 patients we did not detect any additional viral variants harboring major resistance mutations. Only in patient B, for whom a virus containing a single T69N change was identified by population sequencing, additional mutations were detected with UDPS: a mixture of T69D/N/A (2.6, 12.3, 1.7% respectively) and variants containing K103N (7.4%). For patient C the initial sample containing M184V was not available for UDPS. In a sample drawn six months later, the M184V could not be detected by UDPS (Table 1).

In some patients (A, F, H) additional minority wild type codons were detected with UDPS at positions where population sequencing found a drug resistant mutation. At position 215, atypical and intermediate variants are frequently observed by population sequencing. In none of the patients with atypical or intermediate codons at position 215 (S/E/L), additional resistant variants Y or F were found.

Treatment response

HAART was initiated in all patients and clinical follow-up was monitored for 3-69 months (median: 33 months) as is summarized in Table 1. The majority of the patients is currently still under treatment in our medical center. Patient J participated in a study for which he interrupted therapy after 6 months. Patients A and C were lost to follow-up after 3 and 14 months of treatment. The backbone of all therapy regimens consisted of tenofovir with emtricitabine. In addition to the NRTI backbone, eight patients started with a non-nucleoside reverse transcriptase inhibitor (NNRTI)-, one with a ritonavir-boosted protease inhibitor (PI/r) and one patient both PI/r and NNRTI. A rapid and consistent decline in plasma HIV RNA was observed in nine of ten patients (Figure 1). Patient B, the patient with minority resistance mutations detected by UDPS, was the only patient who experienced virological failure. Indeed, preexisting resistant variants with a K103N and variants at position 69 became the dominant species during virological failure.

Table 1. Baseline characteristics, therapy and genotypic analyses.

Patient	HIV-1 diagnosis (last negative test)	CD4 count at diagnosis (cells/µl)	Therapy: TDF + FTC/3TC and:	Follow-up on therapy (months)	HIV-RNA sequencing sample (copies/ml)	Genotypic analysis						
						Population-based	UDPS	% variant	Reads	Additional resistance mutations detected using UDPS	% variant	Reads
A	July 2007 (2005)	470	EFV	3	>100.000	M41L	41L 41M	85 15	2623	None		
B[1]	March 2007 (n.a.)	30	EFV	36	>100.000	T69NT	69N 69T	12 83	2516	69 A 69D	2 3	2516
										103N 103K	7 93	2967
C[2]	January 2003 (2001)	490	NVP	14	>100.000	M184VM	184V 184 M	<1 100	3277	None		
D	February 2005 (2001)	370	EFV	69	>100.000	T215E	215E 215T	95 <1	2582	None		
E	May 2008 (2007)	210	EFV	32	>100,000	T215E	215E 215T	97 <1	2115	None		
F	December 2007 (2003)	30	EFV	40	>100.000	T215L	215L 215T	98 1	2613	None		
G	July 2007 (n.a.)	110	EFV → ATV/r	44	>100.000	T215S	215S 215T	99 <1	2246	None		
H	January 2007 (n.a.)	290	ATV/r	34	>100.000	T215S	215S 215T	98 1	2685	None		
I	February 2008 (2003)	270	EFV	31	>100.000	T215S	215S 215T	99 <1	2680	None		
J	April 2008 (2008)	200	EFV, LPV/r → EFV	6	>100.000	K219Q	219Q 219K	100 <1	2562	None		

UDPS was performed on the same sample as population-based analysis, except for two cases in which the sample used for UDPS was taken [1] 2 weeks or [2] 6 months later than the sample used for population-based sequencing. If available, the year of the last negative test is indicated in parentheses in the second column. Therapy switch is indicated with an arrow. N.a.: not available, FTC: Emtricitabine, 3TC: Lamivudine, TDF: Tenofovir, EFV: Efavirenz, NVP: Nevirapine, ATV/r: Ritonavir-boosted Atazanavir, LPV/r: Ritonavir-boosted Lopinavir.

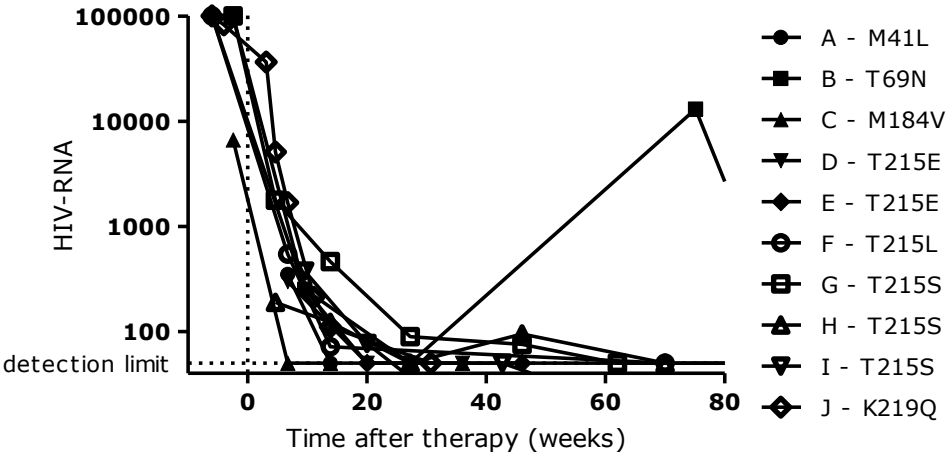


Figure 1. Virological outcome of the first 80 weeks of follow-up. For all patients virological outcome was monitored. During the follow-up, the method of HIV-RNA determination changed from Cobas Amplicor 1.5 to Cobas Taqman 2.0 (both Cobas Ampliprep, Almere, the Netherlands). For this graph, a lower detection limit of 50 copies/ml was used.

Discussion

We studied the viral quasispecies and therapy response in ten MSM who were diagnosed with a single transmitted NRTI-related drug resistance mutation (singleton). The objective of this study was to gain more insight into the relationship between singletons found at HIV diagnosis by population sequencing and the possible presence of additional resistance minority mutations. We used UDPS and therapy response to gain more insight into the possible presence of additional minority variants. Using UDPS we did not find additional minority resistance mutations in plasma from nine out of ten patients. Despite the use of low genetic barrier NNRTI-based regimens in most patients, no resistant viruses emerged in these nine patients.

In the one patient that experienced therapy failure, we detected additional minority resistance mutations, K103N and a mixture at position 69, before therapy was initiated. This minority population became the dominant population during therapy and was most likely responsible for virological failure.

Similar results were described in a report focusing on position 215 in reverse transcriptase. Using UDPS, no minority variants containing T215Y or F were detected in 22 patients infected with viral variants harboring atypical or revertant mutations at this codon[12]. Unfortunately this study did not report on therapy outcome. Several papers describe a higher risk of virological failure in therapy

naïve patients on an NNRTI-containing regimen when transmitted minority NNRTI mutations are found[5, 11, 13, 14]. The efficacy of NNRTIs is strongly affected by even a single mutation, whereas a combination of tenofovir and 3TC/FTC retains most of its activity in the setting of a transmitted single NRTI mutation.

We hypothesize that the absence of viruses with more extensive resistance profiles in these newly diagnosed individuals may be explained by circulation of viruses containing stable singletons. In treated individuals experiencing therapy failure, viruses containing complex resistance profiles can be selected[15] and subsequently transmitted. In the newly infected individual, complex resistance profiles may partially revert in the absence of drug pressure resulting in (stable) singletons[16]. Onward transmission of these stable singletons by therapy naïve patients may have become an important source fuelling transmission of drug resistance. This is supported by the findings of large phylogenetic studies that indicate a major role of onward transmission during early HIV infection for the spread of HIV[17, 18].

Our data indicate that detection of a single NRTI mutation by population sequencing does not always imply the presence of clinically relevant additional resistance mutations. In clinical practice, many patients diagnosed with a singleton NRTI are treated with a high genetic barrier therapy containing a boosted PI. Our results, although the number of patients is small, suggest that a tenofovir based NNRTI regimen may be an effective alternative. Larger studies are warranted to determine the best treatment strategies for patients with transmitted NRTI singletons.

Acknowledgements and funding

We would like to acknowledge T. Mes from Erasmus Medical Center, Rotterdam, the Netherlands and B. Hanczaruk, T. E, Arnold from 454 life sciences, a Roche company, Bradford, CT, USA for excellent technical assistance. We also thank Jan Hollander, MD, for providing patient details.

This work was supported by the Dutch Aids Fonds [grant number 2007034]. Birgitte B. Simen is an employee of 454 Life Sciences, a Roche Company, which performed the ultra-deep pyrosequencing free of charge.

References

1. Wheeler WH, Ziebell RA, Zabina H et al. Prevalence of transmitted drug resistance associated mutations and HIV-1 subtypes in new HIV-1 diagnoses, U.S.-2006. *AIDS* 2010; **24**: 1203-12.
2. Little SJ, Holte S, Routy JP et al. Antiretroviral-drug resistance among patients recently infected with HIV. *N Engl J Med* 2002; **347**: 385-94.

3. SPREAD. Transmission of drug-resistant HIV-1 in Europe remains limited to single classes. *AIDS* 2008; **22**: 625-35.
4. Toni TA, Asahchop EL, Moisi D et al. Detection of human immunodeficiency virus (HIV) type 1 M184V and K103N minority variants in patients with primary HIV infection. *Antimicrob Agents Chemother* 2009; **53**: 1670-2.
5. Johnson JA, Li JF, Wei X et al. Minority HIV-1 drug resistance mutations are present in antiretroviral treatment-naive populations and associate with reduced treatment efficacy. *PLoS Med* 2008; **5**: e158.
6. Pingen M, Nijhuis M, de Bruijn JA et al. Evolutionary pathways of transmitted drug-resistant HIV-1. *J Antimicrob Chemother* 2011; **66**: 1467-80.
7. Van Laethem K, De Munter P, Schrooten Y et al. No response to first-line tenofovir+lamivudine+efavirenz despite optimization according to baseline resistance testing: impact of resistant minority variants on efficacy of low genetic barrier drugs. *J Clin Virol* 2007; **39**: 43-7.
8. Ghosn J, Pellegrin I, Goujard C et al. HIV-1 resistant strains acquired at the time of primary infection massively fuel the cellular reservoir and persist for lengthy periods of time. *AIDS* 2006; **20**: 159-70.
9. Li JZ, Paredes R, Ribaudo HJ et al. Low-frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: a systematic review and pooled analysis. *JAMA* 2011; **305**: 1327-35.
10. Bennett DE, Camacho RJ, Otelea D et al. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. *PLoS One* 2009; **4**: e4724.
11. Simen BB, Simons JF, Hullsiek KH et al. Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. *J Infect Dis* 2009; **199**: 693-701.
12. Mitsuya Y, Varghese V, Wang C et al. Minority human immunodeficiency virus type 1 variants in antiretroviral-naive persons with reverse transcriptase codon 215 revertant mutations. *J Virol* 2008; **82**: 10747-55.
13. Halvas EK, Wiegand A, Boltz VF et al. Low frequency nonnucleoside reverse-transcriptase inhibitor-resistant variants contribute to failure of efavirenz-containing regimens in treatment- experienced patients. *J Infect Dis* 2010; **201**: 672-80.
14. Paredes R, Lalama CM, Ribaudo HJ et al. Pre-existing minority drug-resistant HIV-1 variants, adherence, and risk of antiretroviral treatment failure. *J Infect Dis* 2010; **201**: 662-71.
15. Richman DD, Morton SC, Wrin T et al. The prevalence of antiretroviral drug resistance in the United States. *AIDS* 2004; **18**: 1393-401.
16. Pingen M, Nijhuis M, de Bruijn JA et al. Evolutionary pathways of transmitted drug-resistant HIV-1. *J Antimicrob Chemother* 2011.
17. Brenner BG, Roger M, Routy JP et al. High rates of forward transmission events after acute/early HIV-1 infection. *J Infect Dis* 2007; **195**: 951-9.
18. Yerly S, Vora S, Rizzardi P et al. Acute HIV infection: impact on the spread of HIV and transmission of drug resistance. *AIDS* 2001; **15**: 2287-92.

Chapter 7

Infection with the Frequently Transmitted M41L Variant Has No Impact on Selection of Tenofovir Resistance

Marieke Pingen[1,2], Monique Nijhuis[1], Tania Mudrikova[3], Arjan van Laarhoven[1], Nienke Langebeek[4], Clemens Richter[4], Charles A.B. Boucher[2] and Annemarie M. J. Wensing[1].

[1] Virology, Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, the Netherlands;
[2] Department of Virology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands;
[3] Department of Internal Medicine and Infectious Diseases, University Medical Center Utrecht Utrecht, the Netherlands;
[4] Department of Internal Medicine, Rijnstate Hospital, Arnhem, the Netherlands.

Submitted

Abstract

Objective In approximately 10% of newly diagnosed HIV-1 patients, viral variants harboring drug resistance mutations are detected. The M41L mutation in reverse transcriptase (RT) is one of the most frequently observed transmitted drug resistance mutations. We investigated the impact of a single M41L at baseline on selection of resistance against the first-line drugs tenofovir (TDF) and emtricitabine (FTC) *in vivo* and *in vitro*.

Design Retrospective observational cohort study including 265 patients on a first-line regimen containing TDF and FTC and extensive *in vitro* investigation of selection of drug resistance by TDF and FTC.

Methods Virological outcome of a regimen containing TDF and FTC in patients diagnosed with an HIV-1 variant harboring M41L (n=17) was compared to patients infected with wild type virus (n=248). *In vitro* selection of drug resistance under drug pressure with TDF and/or FTC was investigated for wild type virus and M41L-variants.

Results Patients diagnosed with HIV-1 harboring a single M41L mutation had a comparable virological response on first-line therapy with TDF compared to patients diagnosed with wild type HIV-1. *In vitro*, the presence of a single M41L impacted neither the susceptibility to TDF nor the selection of resistance to TDF and/or FTC. The predominantly selected resistance mutations were M184V/I for FTC and K65R for TDF and TDF+FTC.

Conclusions The presence of the RT M41L mutation in therapy naïve individuals does not impact the mutational profile selected by TDF. Therefore, a low-genetic barrier regimen may be initiated when M41L is present as a single resistance mutation at baseline.

Introduction

Approximately 10% of all newly diagnosed HIV-1 patients in Europe and the USA harbor a viral variant with at least one drug resistance mutation. The reverse transcriptase (RT) M41L mutation is one of the most frequently observed transmitted resistance mutations with a prevalence of 20-31% among patients diagnosed with drug resistant HIV-1[1-3].

M41L belongs to the group of thymidine analogue mutations (TAMs) (M41L, D67N, K70R, L210W, T215Y/F, K219E/Q) which are selected by the nucleoside reverse transcriptase inhibitors (NRTIs) zidovudine and stavudine, which have been used extensively[4, 5]. TAMs do not only decrease susceptibility to zidovudine and stavudine, but also lead to cross-resistance to other NRTIs such as tenofovir (TDF). Clinical studies have shown that the presence of three or more TAMs, in particular patterns with M41L or L210W, impairs virological efficacy of TDF[6-8]. In patients infected with wild type virus, TAMs are rarely observed upon virological failure of TDF-containing first-line regimens. If resistance to TDF is selected, mutations K65R and/or K70E are primarily detected[9-13].

Little is known about the biological and clinical consequences of transmitted HIV-variants harboring a single M41L in relation to the use of TDF. The presence of a single M41L at baseline does not lead to an evidently decreased susceptibility to TDF *in vitro*[14]. However, it remains unknown whether the presence of M41L decreases the genetic barrier to resistance or skews the resistance pathway towards accumulation of TAMs in case of virological failure. As the TDF and emtricitabine (FTC) co-formulation Truvada® is the most frequently used first-line NRTI backbone in clinical practice, concerns for rapid selection of TAMs may result in the use of more complex initial regimens with a higher genetic barrier. However, this leads to an increased pill burden, elevated costs, and may increase toxicity. Insight into the effect of M41L at baseline on selection of resistance by TDF/FTC may improve first-line treatment options for patients. Furthermore, the FDA recently approved the use of TDF/FTC for pre-exposure prophylaxis (PrEP) to prevent HIV-1 transmission in high-risk individuals. Considering the frequent presence of the M41L mutation in transmitted HIV-1, it is essential to know whether this mutation could possibly influence the efficacy of TDF/FTC PrEP. We investigated the impact of a single M41L at baseline by studying therapy response to TDF/FTC *in vivo* and the selection of drug resistance to TDF and/or FTC *in vitro*.

Methods and Materials

Study population

We investigated individuals who were newly diagnosed with HIV-1 in two centers in the Netherlands, University Medical Center Utrecht and Rijnstate Hospital in

Arnhem. All patients were participants of the Dutch ATHENA Cohort. Inclusion criteria were: 1) baseline genotypic resistance test generated between 2007 and 2010, 2) initiation of first-line therapy with a TDF/FTC-containing regimen, 3) at least one year of clinical follow-up after therapy initiation, and 4) detection of wild type virus or a variant harboring M41L as the only transmitted resistance mutation according to the IAS-USA list of resistance mutations[15].

Clinical outcome

Low-genetic barrier antiretroviral therapy regimens were defined as TDF/FTC with a non-nucleoside reverse transcriptase inhibitor (NNRTI) or raltegravir as a third drug. Regimens that contained either a boosted protease inhibitor or >3 drugs were classified as high-genetic barrier regimens. Virological failure during the first year of treatment was defined as: 1) not achieving HIV-RNA <50 copies/ml, or 2) HIV RNA >200 copies/ml after achieving HIV RNA <50 copies/ml. The analyses were conducted on an intent-to-treat basis. Statistical analysis was conducted using Fisher's exact test (categorical data) or Mann-Whitney U test (numerical data) in SPSS Statistics 19 (IBM).

Phylogenetic analysis

Baseline population sequences of the HIV *pol* gene from all patients included in the cohort analysis were aligned using ClustalW. To avoid bias caused by the presence of M41L, amino acid position 41 in RT was removed from the alignment. A maximum-likelihood phylogenetic tree was calculated with MEGA 5.05 using the general time-reversing model of nucleotide substitution with Γ-distributed rate heterogeneity with 1000 bootstrap replicates.

Virus panel

Three subtype B M41L-viruses were generated: two patient-derived M41L viruses (pM41L-1 and -2) and a site-directed mutant (SDM-M41L). One virus was selected from the large transmission cluster (Figure 1). To ensure results were not specific for the genetic background within that particular cluster, a second isolate with a single M41L that did not cluster was chosen (data not shown). The N-terminus of RT (amino acids 25-314) of both patient-derived viruses were amplified, digested and ligated using the previously described NRT-vector system[16]. The M41L mutation was introduced by site-directed mutagenesis in wild type HXB2 (primer M41L-RT: 5'-ATT TGT ACA GAG CTG GAA AAG GAA G-3', nucleotides 2658-2682) using the aforementioned vector system. Clones were verified by sequence analysis. Virus stocks were obtained by transfection of HEK293T cells with plasmid DNA using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. $TCID_{50}$ was determined by end-point dilution in MT2 cells (baseline viruses) or SupT1 cells (*in vitro* selection viruses).

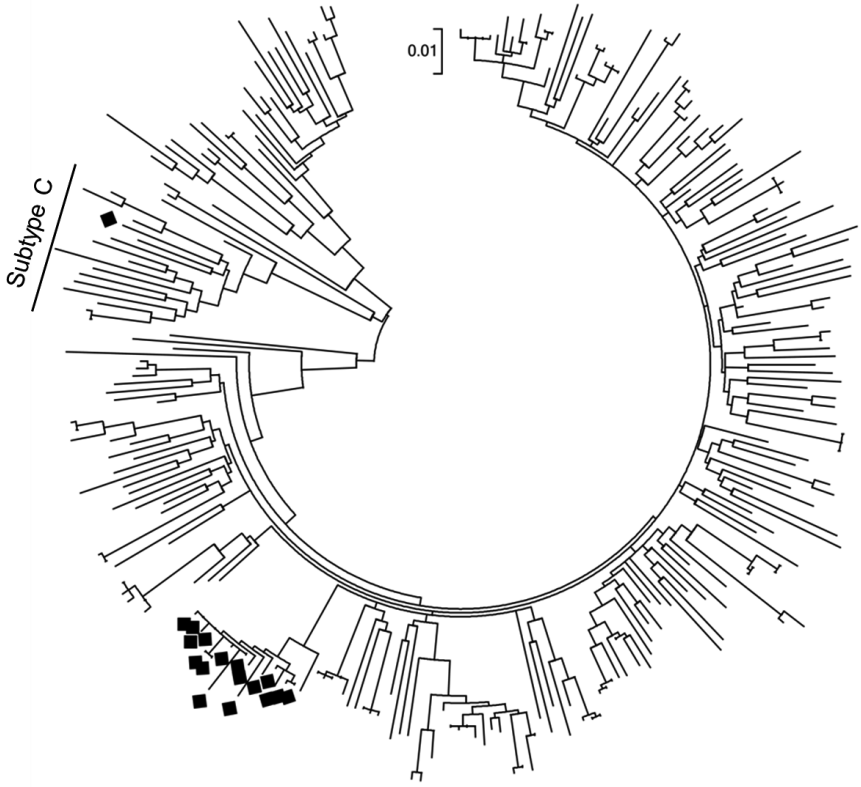


Figure 1. Maximum likelihood phylogenetic tree of baseline sequences. The first available sequence of all patients included in the cohort analysis (n = 265) was used to calculate a maximum likelihood tree. Sequences harboring M41L in RT are indicated with a square.

In vitro drug selection and evolution

A checkerboard approach was used to determine the drug concentration of TDF in combination with FTC. SupT1 cells were inoculated with wild type (HXB2) at a MOI of 0.001 for two hours before a range of FTC (0-0.9 μM) and TDF (0-2.0 μM) was added. Cells were monitored regularly for cytopathic effect (CPE). Twice a week, half of the culture was replaced by culture media with the same drug concentrations. The highest drug concentrations of the cultures in which CPE developed after two weeks were used in the first passage of the *in vitro* selection experiments. The initial FTC concentration in the *in vitro* selection experiments of 0.011 μM FTC was below the 50% effective concentration (EC_{50}) in SupT1 cells, while the initial TDF concentration of 2 μM TDF was above the EC_{50}.

In vitro selections were started in parallel five-fold cultures in which $2x10^5$ SupT1 cells were infected with an MOI of 0.001. The initial drug concentration

was 0.011 μM FTC, 4 μM TDF or 0.011 μM FTC + 2 μM TDF. Cultures were regularly monitored for CPE and replenished twice a week with fresh culture media supplemented with drug(s). When full-blown CPE was observed, cell-free supernatant was harvested and stored at -80°C. Subsequent passages were started by infecting $2x10^5$ SupT1 cells with 10-50 μl virus of the previous passage. Drug concentrations were gradually increased to 0.352 μM FTC, 32 μM TDF or 0.132 μM FTC + 24 μM TDF. After five (TDF and TDF/FTC) or six passages (FTC), viral RNA was isolated for genotypic analysis of the entire RT gene. Increased drug levels of TDF and/or FTC repeatedly inhibited viral replication in several of the wild type and M41L cultures which were therefore discontinued. All viruses were propagated in five-fold for ten passages in the absence of drugs as a control. This revealed persistence of M41L in all cultures for 10 passages. No additional resistance-related mutations were selected in any of the cultures (Supplementary Table 1).

Phenotypic drug susceptibility

Viruses resulting from the *in vitro* selection experiments were expanded by infecting $2x10^6$ SupT1 cells in the absence of drugs. The *in vitro* drug susceptibility of the virus panel was determined using a multiple cycle assay[17]. Resistance was defined as fold increase in EC_{50} compared to wild type. At least two independent experiments were performed to calculate phenotypic drug resistance. The susceptibility of all viruses with a single M41L to TDF and FTC were comparable to wild type virus (TDF EC_{50}: 1.9 μM, FTC EC_{50}: 1.6 μM in MT2 cells) (Supplementary Table 2).

Results

Transmission of HIV variants containing M41L

All patients from whom a baseline genotypic analysis was performed between 2007 and 2010 were enrolled in this study. Of 530 patients, 279 initiated TDF/FTC-containing first-line regimens and had at least one year clinical follow-up. Of these, 31 patients were diagnosed with drug resistant HIV-1, of which 17 patients possessed a variant harboring only M41L. The remaining 248 patients were diagnosed with wild type virus. The latter two groups were included in the analysis (Figure 2).

Of the 265 patients included, 90% were male, and the main route of transmission was MSM contact (72%). Most patients were infected with HIV-1 subtype B (80%). At baseline, the median CD4 count was 290 cells/mm^3 and median log plasma HIV-RNA was 5.1 copies/ml. Except for a slightly higher HIV-RNA (5.5 vs 5.1, $p = 0.033$), the baseline characteristics of patients diagnosed with a M41L variant were comparable to patients with a wild type virus (Table 1).

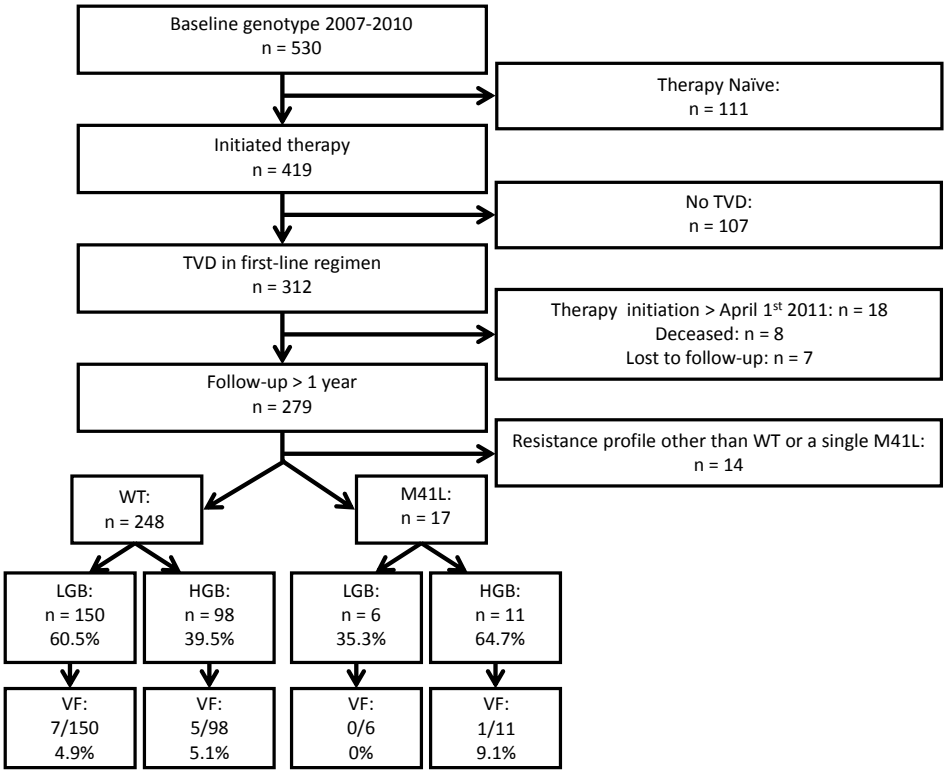


Figure 2. Inclusion of patients and clinical outcome of cohort analysis. Abbreviations: WT: Wild type virus, M41L: Variant harboring M41L as only resistance mutation, LGB: Low-genetic barrier regimen (truvada with a efavirenz, nevirapine or raltegravir), HGB: High-genetic barrier regimen (truvada with a boosted protease inhibitor or regimens containing >3 drugs), VF: virological failure.

Phylogenetic analysis of the viruses isolated from all patients included (n=265) revealed that 16/17 variants harboring M41L belonged to a large transmission cluster (Figure 1, bootstrap value 98%). These 16 clustering viruses were subtype B, whereas the non-clustering virus was subtype C. Although the two participating centers are located in distinct regions of the Netherlands, viruses from both centers frequently clustered, indicating highly interlacing epidemics.

Clinical impact of a transmitted HIV-1 variants harboring M41L

The majority of patients continued the initial first-line regimen during follow-up. Only 23/265 patients (8.7%) (temporarily) stopped TDF/FTC treatment or switched to other NRTIs, mostly as a result to toxicity (n=7) or patient decision to discontinue treatment (n=6). To assess whether detection of a single M41L in the baseline genotype influenced the choice of first-line therapy, the initial regimens of patients diagnosed with M41L-variant or wild type virus were compared. The

Table 1. Patient characteristics

		Total n = 265	**Wild type** n = 248	**M41L** n = 17
Age median (IQR)		39.6 (31.6-47.8)	39.6 (31.2-48.4)	39.4 (28.9-43.9)
Male gender n (%)		238 (89.8)	221 (89.1)	17 (100)
Transmission route n (%)	**MSM**	190 (71.7)	173 (69.8)	17 (100)
	HSX	58 (21.5)	58 (23.4)	0 (0)
	Other/unknown	17 (6.4)	17 (6.9)	0 (0)
Subtype B n (%)		213 (80.4)	197 (79.4)	16 (94.1)
CD4 cell count median (IQR), cells/mm^3		290 (150-422)	289 (149-423)	337 (151-397)
Plasma HIV-RNA median (IQR) log copies/ml		5.1 (4.8-5.6)	5.1 (4.8-5.5)	5.5 (5.0-5.9)

Abbreviations:, IQR: Interquartile range, MSM: men having sex with men, HSX: heterosexual.

comparison revealed that patients diagnosed with a M41L variant received a high-genetic barrier regimen significantly more often than patients diagnosed with wild type virus (11/17 (64.7%) vs 98/248 (39.5%) Fisher's exact test: p = 0.044).

The rate of virological failure during the first year of treatment was comparable between patients diagnosed with a M41L variant or wild type (1/17 (5.9%) vs. 12/236 (5.1%), p=0.586). Interestingly, none of the patients diagnosed with M41L-variants who initiated a low-genetic barrier regimen experienced virological failure. The rate of virological failure on a low-genetic barrier regimen was not statistically different for patients infected with M41L-variants or wild type virus (0/6 (0.0%) vs 7/150 (4.9%), p=1.000).

In the single patient infected with a M41L variant who experienced therapy failure on a high-genetic barrier regimen resistance testing did not reveal selection of additional NRTI mutations. For seven patients diagnosed with wild type virus, genotypic resistance analysis was successfully performed during virological failure. In one patient who received a high genetic barrier regimen a NRTI mutation (M184V) was selected. In another patient treated with a low-genetic barrier regimen the NNRTI-related mutations K103KN and V106MV were detected in absence of M184V.

Impact of M41L on *in vitro* selection of resistance to TDF and FTC

Impact of the M41L mutation on *in vitro* selection of resistance to TDF and FTC, was assessed by comparison of two patient-derived viruses containing M41L as the only major resistance-related mutation (pM41L-1 and -2), a site-directed mutant (SDM-M41L) and wild type virus. During these experiments the drug pressure was gradually increased and comparable selection rates were observed

for wild type and SDM-M41L, indicating M41L does not affect the rate of selection of resistance mutations against TDF and FTC.

Under TDF pressure alone (Table 2, Supplementary Table 3), the M41L mutation persisted in 5/6 cultures, while no TAMs were selected. For wild type virus as well as M41L-containing viruses, the dominant selected resistance profile was K65R. The sole presence of K65R resulted in a 21-fold resistance against TDF (wild type virus) and 12 to 15-fold when K65R appeared in the background of M41L. Under FTC pressure alone (Table 2, Supplementary Table 4), M41L, if present at baseline, persisted in all cultures and no selection of TAMs was observed. Again, the resistance patterns of wild type and M41L-containing viruses were very similar. In the majority of cultures (15/17), mutations at position 184 were selected. M184V conferred very high levels of phenotypic resistance to FTC (>300-fold) independent of the presence of M41L. The average level of resistance to FTC of a virus harboring M184I was 120-fold. In two cultures K65R was selected, once alone and once in combination with M184V. The effect of K65R on the level of resistance against FTC was independent of the presence of M41L (19 to 21-fold for wild type vs SDM-M41L).

Although all viruses were able to replicate under TDF pressure in combination with FTC during initial passages, viral replication was completely inhibited in the majority of cultures during later passages. Four cultures could be successfully propagated under high drug pressure, and in all four experiments K65R was selected (Table 2, Supplementary Table 5).

Discussion

This study investigated the impact of the M41L mutation present at baseline on the development of resistance during exposure to TDF and FTC. Patients diagnosed with HIV-1 harboring M41L had an excellent virological response after

Table 2. Resistance patterns and drug-susceptibility after *in vitro* selections

	WT (number of cultures)		**Fold resistance**	**M41L (number of cultures)**		**Fold resistance**
TDF	K65R	(2/2)	21x	K65R	(5/6)	12 to 15x
				D67G	(2/6)	4x
FTC	K65R	(1/5)	21x	K65R	(1/12)	10 to 20x
	D67G	(1/5)	n.d.	D67G	(1/2)	5x
	M184I/V	(4/5)	120 to >300x	M184I/V/T	(11/12)	65 to >300x
TDF/FTC				K65R	(4/4)	10 to 20x

Abbreviations: WT: wild type, M41L: Variants containing M41L, TDF: tenofovir, FTC: Emtricitabine, TDF/FTC: combination of tenofovir and emtricitabine.

one year of antiretroviral therapy containing TDF/FTC, even when treated with a low-genetic barrier regimen. Furthermore, the presence of M41L did not affect the selected mutational profile under drug pressure with TDF and/or FTC *in vitro*.

In our cohort of 265 patients, 17 patients were diagnosed with an HIV-1 variant harboring a single M41L mutation, enabling us to investigate the impact of M41L on treatment outcome. Although these are relatively small numbers, this is, to our knowledge, the largest group of patients described with a similar transmitted mutation for whom outcome data is available. Of these 17 patients, 16 achieved and maintained a suppressed HIV-RNA during the first year of therapy, which is similar to the virological outcome of patients infected with wild type virus. Our findings suggest that a TDF/FTC-containing regimen can be efficacious for treatment of HIV-variants harboring a single M41L mutation.

Previous research has focused on viruses containing complex TAM profiles because these were frequently observed in patients experiencing virological failure on thymidine analogues. In contrast, we focused on variants harboring a single M41L as this is the most frequently observed mutation in baseline genotypes of newly diagnosed patients. The detection of variants harboring a single M41L at diagnosis may be due to partial reversion of an extensive TAM profile after transmission from a treated patient to a new host. HIV-1 variants containing an extensive resistance profile may remain present as minority or archived variants, and may rapidly re-emerge after therapy initiation[18]. However, we observed large transmission clusters consisting of viruses with M41L from therapy-naïve individuals[19, 20], which indicates that a large proportion of patients diagnosed with M41L variants may be infected due to onward transmission of HIV-1 variants harboring M41L as a single resistance mutation. In these patients it is unlikely that highly resistant minority variants are present in the quasispecies.

Furthermore, we explored the impact of the frequently transmitted M41L mutation on the selection of drug resistance *in vitro*. Both wild type viruses and viruses harboring M41L mainly selected the mutations K65R or M184V/I during *in vitro* pressure of TDF or FTC. Similar results have been reported by Margot *et al.*[12], who characterized resistance profiles selected under pressure of these drugs for wild type virus alone. This study also showed that *in vitro* selection of resistance against TDF/FTC was strongly dependent on drug concentrations. At high concentrations of TDF and FTC, selection of K65R and M184V was observed. At intermediate concentrations of TDF and FTC, K65R was selected[12], which is in agreement with our results.

It has been shown that the presence of multiple TAMs decreases the ability of K65R to discriminate between NRTIs and the natural nucleoside resulting in a decreased level of resistance [21]. Our data show that the presence of

M41L alone already diminishes the negative effect of K65R on TDF susceptibility. This effect was most pronounced in patient-derived viruses.

Recent evidence indicates that resistance or compensatory mutations related to TAMs or M184 variants can also be selected in the connection domain and RNaseH[22, 23]. Therefore, a possible limitation of this study is that only the N-terminus of RT was used for the generation of patient-derived viruses. However, sequence analysis of the entire RT gene revealed that mutations were selected in the C-terminus in only a minority of the cultures and no changes associated with therapy were observed[22].

Our data has important clinical implications. Our clinical data show that low-genetic barrier regimens may be effective in treating patients diagnosed with a single M41L. This is especially true when the presence of additional TAMs in the quasispecies is unlikely, based on clinical or phylogenetic evidence. Additionally, M41L did not skew the virus towards selection of multiple TAMs. This suggests that the presence of a single M41L does not affect the genetic barrier towards selection of resistance against TDF and/or FTC. Our data indicate that the efficacy of TDF/FTC as PrEP will not be affected by the presence of a single M41L. Even more important, the presence of a single M41L has no major impact on the potential development of additional resistance mutations under PrEP.

In conclusion, the presence of a single M41L at baseline did not impair the virological response to first-line regimens containing TDF nor impact selection of resistance to TDF and/or FTC *in vitro*. Taken together, these results suggest that initiation of a high-genetic barrier regimen may not be required when patients are infected with a drug resistant strain containing M41L as the only resistance mutation.

Acknowledgments and funding

We would like to thank Dorien de Jong and Pascal Mantiri for excellent technical support, Justin E. Swartz for assistance in analysis and Susan C. Aitken for critical reading of the manuscript. This work was supported by the Aids Fonds [grant number 2007034], The Netherlands Organization for Scientific Research [NWO VIDI grant 91796349] and the European Commission [grant number LSHP-CT-2006-518211].

References

1. Wheeler WH, Ziebell RA, Zabina H et al. Prevalence of transmitted drug resistance associated mutations and HIV-1 subtypes in new HIV-1 diagnoses, U.S.-2006. *AIDS* 2010; **24**: 1203-12.
2. Vercauteren J, Wensing AM, van de Vijver DA et al. Transmission of drug-resistant HIV-1 is stabilizing in Europe. *J Infect Dis* 2009; **200**: 1503-8.

3. Programme DFobotS. Recent dynamics of transmitted drug resistance in Europe: SPREAD programme 2006-2007. *9th European Workshop on HIV & Hepatitis Treatment Strategies & Antiviral Drug Resistance*. Paphos, Cyprus: Reviews in Antiviral Therapy & Infectious Diseases, 2011; 15-6.
4. Boucher CA, O'Sullivan E, Mulder JW et al. Ordered appearance of zidovudine resistance mutations during treatment of 18 human immunodeficiency virus-positive subjects. *J Infect Dis* 1992; **165**: 105-10.
5. Larder BA, Kemp SD. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* 1989; **246**: 1155-8.
6. Margot NA, Isaacson E, McGowan I et al. Genotypic and phenotypic analyses of HIV-1 in antiretroviral-experienced patients treated with tenofovir DF. *AIDS* 2002; **16**: 1227-35.
7. Ross LL, Parkin N, Gerondelis P et al. Differential impact of thymidine analogue mutations on emtricitabine and lamivudine susceptibility. *J Acquir Immune Defic Syndr* 2006; **43**: 567-70.
8. Barrios A, de Mendoza C, Martin-Carbonero L et al. Role of baseline human immunodeficiency virus genotype as a predictor of viral response to tenofovir in heavily pretreated patients. *J Clin Microbiol* 2003; **41**: 4421-3.
9. Brenner BG, Oliveira M, Doualla-Bell F et al. HIV-1 subtype C viruses rapidly develop K65R resistance to tenofovir in cell culture. *AIDS* 2006; **20**: F9-13.
10. Gallant JE, DeJesus E, Arribas JR et al. Tenofovir DF, emtricitabine, and efavirenz vs. zidovudine, lamivudine, and efavirenz for HIV. *N Engl J Med* 2006; **354**: 251-60.
11. Margot NA, Lu B, Cheng A et al. Resistance development over 144 weeks in treatment-naive patients receiving tenofovir disoproxil fumarate or stavudine with lamivudine and efavirenz in Study 903. *HIV Med* 2006; **7**: 442-50.
12. Margot NA, Waters JM, Miller MD. In vitro human immunodeficiency virus type 1 resistance selections with combinations of tenofovir and emtricitabine or abacavir and lamivudine. *Antimicrob Agents Chemother* 2006; **50**: 4087-95.
13. Sluis-Cremer N, Sheen CW, Zelina S et al. Molecular mechanism by which the K70E mutation in human immunodeficiency virus type 1 reverse transcriptase confers resistance to nucleoside reverse transcriptase inhibitors. *Antimicrob Agents Chemother* 2007; **51**: 48-53.
14. Rhee SY, Taylor J, Wadhera G et al. Genotypic predictors of human immunodeficiency virus type 1 drug resistance. *Proc Natl Acad Sci U S A* 2006; **103**: 17355-60.
15. Johnson VA, Calvez V, Gunthard HF et al. 2011 update of the drug resistance mutations in HIV-1. *Top Antivir Med* 2011; **19**: 156-64.
16. van Maarseveen NM, Huigen MC, de Jong D et al. A novel real-time PCR assay to determine relative replication capacity for HIV-1 protease variants and/or reverse transcriptase variants. *J Virol Methods* 2006; **133**: 185-94.
17. Boucher CA, Keulen W, van Bommel T et al. Human immunodeficiency virus type 1 drug susceptibility determination by using recombinant viruses generated from patient sera tested in a cell-killing assay. *Antimicrob Agents Chemother* 1996; **40**: 2404-9.
18. Li JZ, Paredes R, Ribaudo HJ et al. Low-frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: a systematic review and pooled analysis. *JAMA* 2011; **305**: 1327-35.
19. Pingen M, van Laarhoven A, Mudrikova T et al. Unexpected high prevalence of transmitted drug resistance among newly diagnosed patients in the central region of the Netherlands. *7th European HIV Drug Resistance Workshop*. Stockholm, Sweden, 2009.

20. Lindstrom A, Ohlis A, Huigen M et al. HIV-1 transmission cluster with M41L 'singleton' mutation and decreased transmission of resistance in newly diagnosed Swedish homosexual men. *Antivir Ther* 2006; **11**: 1031-9.
21. Parikh UM, Zelina S, Sluis-Cremer N et al. Molecular mechanisms of bidirectional antagonism between K65R and thymidine analog mutations in HIV-1 reverse transcriptase. *AIDS* 2007; **21**: 1405-14.
22. von Wyl V, Ehteshami M, Symons J et al. Epidemiological and biological evidence for a compensatory effect of connection domain mutation N348I on M184V in HIV-1 reverse transcriptase. *J Infect Dis* 2010; **201**: 1054-62.
23. Lengruber RB, Delviks-Frankenberry KA, Nikolenko GN et al. Phenotypic characterization of drug resistance-associated mutations in HIV-1 RT connection and RNase H domains and their correlation with thymidine analogue mutations. *J Antimicrob Chemother* 2011; **66**: 702-8.

Supplementary Table 1. Mutation patterns of the virus panel after *in vitro* evolution

	3	41	60	69	112	122	135	162	166	172	178	202	208	211	213	214	245	248	263	272	275	276	293	297	298	312	322	375	431	545
Wild type	S	M	V	T	G	E	I	S	K	R	I	I	H	R	G	L	V	E	K	P	K	V	I	E	E	E	S	I	K	E
SDM-M41L	•	L	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVE-1	•	L	•	**N/T**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVE-2	•	L	•	•	**S/G**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVE-3	•	L	•	•	•	•	•	•	•	**K/R**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVE-4	•	L	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	**L/I**	•	•
IVE-5	•	L	•	•	•	•	•	**S/G**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
pM41L-1	•	L	•	D	•	K	V	C	•	•	L	•	•	T	•	F	M	N	•	•	•	•	•	•	•	•	•	•	•	•
IVE-1	**N**	L	•	D	•	K	V	C	•	•	L	**I/L**	•	T	•	F	M	N	•	•	•	**I/V**	•	•	?	•	•	•	•	•
IVE-2	•	L	•	D	•	K	V	C	•	•	L	•	•	T	**R**	F	M	N	•	•	•	•	•	•	•	•	•	•	•	•
IVE-3	•	L	•	D	•	K	V	C	•	•	L	•	**Y**	T	•	F	M	N	•	•	•	•	•	•	•	•	•	•	•	**E/K**
IVE-4	•	L	•	D	•	K	V	C	•	•	L	•	•	T	**K/R/E/G**	F	M	N	•	•	•	•	•	•	•	•	•	•	**R/K**	•
IVE-5	•	L	•	**D/H**	•	K	V	C	•	•	L	•	•	T	•	F	I/M	N	•	•	•	•	•	•	**E/G**	**E/G**	•	•	•	•
pM41L-2	•	L	I	•	•	•	T	C	R	•	•	•	•	G	•	F	•	•	•	A	T	•	V	V	•	•	•	•	•	•
IVE-1	•	L	I	•	•	•	T	**Y**	R	•	•	•	•	G	•	F	•	•	•	A	T	•	V	V	•	•	T	•	•	•
IVE-3	•	L	I	•	•	•	T	**Y**	R	•	•	•	•	G	•	F	•	•	•	A	T	•	V	V	•	•	•	•	•	•
IVE-4	•	L	I	•	•	•	**T/I**	**Y**	R	•	•	•	•	G	•	F	•	•	•	A	T	•	V	V	•	**E/K**	•	•	•	•

IVE: *in vitro* evolution culture. Columns in grey indicate resistance positions; bold indicates a change during *in vitro* evolution experiment.

Supplementary Table 2. Drug-susceptibility to TDF and FTC for our virus panel and several *in vitro*-selected viruses

Viruses	Drug culture	IAS mutations	Average fold resistance compared to wild type			
Before *in vitro* selections			**TDF**	**(sd)**	**FTC**	**(sd)**
SDM-M41L		M41L	1.3	(0.3)	0.7	(0.1)
pM41L-1		M41L	1.1	(0.1)	0.6	(0.2)
pM41L-2		M41L	1.5	(0.1)	1.4	(0.1)
After *in vitro* selections			**TDF**	**(sd)**	**FTC**	**(sd)**
wild type	TDF IVS-4	K65R	21.0	(7.3)	21.2	(8.7)
SDM-M41L	TDF IVS-1	M41L, K65R	14.9	(0.1)	19.4	(1.1)
pM41L-1	TDF IVS-5	M41L, K65R	14.1	(1.2)	12.7	(6.1)
pM41L-2	TDF IVS-5	M41L, K65R	12.5	(1.5)	9.9	(1.8)
pM41L-1	**TDF IVS-1**	**M41L, N67G**	**4.4**	**(0.5)**	**4.7**	**(1.8)**
Wild type	FTC IVS-1	M184I	2.2	(1.1)	122.2	(36)
SDM-M41L	FTC IVS-2	M41L, M184I	2.3	(0.9)	65.1	(35)
Wild type	FTC IVS-4	M184V	2.8	(0.5)	>300	(n.a.)
SDM-M41L	FTC IVS-5	M41L, M184V	2.2	(0.7)	>300	(n.a.)

All values are the average of at least two independent experiments. Abbreviations: TDF: Tenofovir, FTC: Emtricitabine, IVS: *In vitro* selection, sd: standard deviation, n.a.: not applicable.

Supplementary Table 3. Mutation patterns of the virus panel after *in vitro* selections with TDF

	41	60	65	67	122	135	162	166	178	179	211	214	218	245	248	254	272	275	293	297	322	360	451	466
Wild type	M	V	K	D	E	I	S	K	I	V	R	L	D	V	E	V	P	K	I	E	S	A	K	V
IVS-1	•	•	**R/K**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVS-4	•	•	**R**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
SDM-M41L	L	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVS-3	L	•	**R**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
pM41L	L	•	•	•	K	V	C	•	L	•	T	F	•	M	N	•	•	•	•	•	•	•	•	•
IVS-1	L	•	•	**G**	K	•	C	•	L	•	T	F	•	M	N	•	•	•	•	•	•	•	•	**V/F**
IVS-2	L	•	**R/K**	**G/D**	K	V	C	•	L	**V/I**	T	F	**N/D**	M	N	•	•	•	•	•	•	•	•	•
IVS-5	L	•	**R**	•	K	V	C	•	L	•	T	F	•	M	N	**I/V**	•	•	•	•	•	•	**K/N**	•
pM41L-2	L	I	•	•	•	T	C	R	•	•	G	F	•	•	•	•	A	T	V	V	•	•	•	•
IVS-4	**M/L**	I	**R/K**	•	•	T	C	R	•	•	G	F	•	•	•	•	A	•	V	V	T	•	•	•
IVS-5	L	I	**R**	•	•	T	**Y**	R	•	•	G	F	•	•	•	•	A	T	V	V	T	**T/A**	•	•

TDF: tenofovir, IVS: *in vitro* selection culture. Columns in grey indicate resistance positions; bold indicates a change during *in vitro* selection experiment.

Supplementary Table 4. Mutation patterns of the virus panel after *in vitro* selections with FTC

	3	16	41	60	65	67	75	118	122	123	135	162	166	172	178	184	204	211	214	221	245	248	272	275	293	297	322	336	372	502	519
Wild type	S	M	M	V	K	D	V	V	E	D	I	S	K	R	I	M	E	R	L	H	V	E	P	K	I	E	S	Q	V	A	N
IVS-1	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	**I**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVS-2	•	•	•	•	•	**G/D**	•	•	•	•	•	•	•	•	•	**I**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVS-3	•	•	•	•	**R/K**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	**V/A**	•
IVS-4	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	**V**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVS-5	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	**I**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
SDM-M41L	•	•	L	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVS-1	•	•	L	•	•	•	•	•	•	•	•	•	•	•	•	**V/I/M**	**K/E**	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVS-2	•	•	L	•	•	•	•	•	•	•	•	•	•	•	•	**I**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVS-3	•	•	L	•	•	•	•	•	•	**D/G**	•	•	•	•	•	**I**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	**Y/N**
IVS-4	•	•	L	•	•	•	•	•	•	•	•	•	•	•	•	**V/I/M**	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
IVS-5	•	•	L	•	•	•	I	•	•	•	•	•	•	•	•	**V**	•	•	•	**H/L**	•	•	•	•	•	•	•	•	•	•	•
pM41L-1	•	•	L	•	•	•	•	•	K	•	V	C	•	•	L	•	•	T	F	•	M	N	•	•	•	•	•	•	•	•	•
IVS-1	•	•	L	•	•	**G/D**	•	**V/A**	K	•	V	C	•	•	L	•	•	T	F	•	M	N	•	•	•	•	•	•	•	•	•
IVS-2	•	•	L	•	**R/K**	•	•	•	K	•	V	C	•	•	L	**V**	•	T	F	•	M	N	•	•	•	•	•	**Q/H**	•	•	•
IVS-3	I	•	L	•	•	•	•	•	K	•	V	C	•	•	L	**V**	•	T	F	•	M	N	•	•	•	•	•	•	•	•	•
IVS-4	•	•	L	•	•	•	•	•	K	•	V	C	•	•	L	**T**	•	T	F	•	M	N	•	•	•	•	•	•	•	•	•
IVS-5	•	•	L	•	•	•	•	•	K	•	V	C	•	•	L	**I**	•	T	F	•	M	N	•	•	•	•	•	•	•	•	•
pM41L-2	•	•	L	I	•	•	•	•	•	•	T	C	R	•	•	•	•	G	F	•	•	•	A	T	V	V	T	•	•	•	•
IVS-2	•	•	L	I	•	•	•	•	•	•	T	Y	R	•	•	**I**	•	G	F	•	•	•	A	T	V	V	T	•	•	•	•
IVS-3	•	•	L	I	•	•	•	•	•	•	T	Y	R	•	•	**V**	•	G	F	•	•	•	A	T	V	V	T	•	•	•	•

FTC: Emtricitabine, IVS: *in vitro* selection culture. Columns in grey indicate resistance positions; bold indicates a change during in vitro selection experiment.

Supplementary Table 5. Resistance patterns of the virus panel after *in vitro* selections with FTC and TDF

	16	41	65	75	122	135	162	178	211	214	245	248
Wild type	M	M	K	V	E	I	S	I	R	L	V	E
SDM-M41L	•	L	**R**	•	•	•	•	•	•	•	•	•
pM41L-1	•	L	•	•	K	V	C	L	T	F	M	N
IVS-1	•	L	**R**	**I**	K	V	C	L	T	F	M	N
IVS-3	•	L	**R**	•	K	V	C	L	T	F	M	N
IVS- 5	**I**	L	**R**	•	K	V	C	L	T	F	M	N

TDF: tenofovir, IVS: *in vitro* selection culture. Columns in grey indicate resistance positions; bold indicates a change during *in vitro* selection experiment.

Chapter 8

Therapy Failure Resulting From Superinfection by a Drug-Resistant HIV Variant

Marieke Pingen[1,2], Jan L. Nouwen[3], Sander Dinant[3], Jan Albert[4,5], Mattias Mild[4], Johanna Brodin[4], Birgitte B. Simen[6], Susan Walsh[7], Manfred Kayser[7], Marchina E. van der Ende[3], Martin Schutten[1] & Charles A.B. Boucher[1]

[1] Department of Virology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands;
[2] Department of Virology, Medical Microbiology, University Medical Center Utrecht, Utrecht, the Netherlands;
[3] Infectious diseases, Erasmus MC, University Medical Center, Rotterdam, the Netherlands;
[4] Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, Stockholm, Sweden;
[5] Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden;
[6] 454 Life Sciences, a Roche Company, Branford, CT, USA;
[7] Department of Forensic Molecular Biology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands.

Antiviral therapy, 2012; 17(8):1621-5.

Abstract

Background HIV-1 infected patients can be superinfected with additional HIV-1 variants. Therapy failure can be the consequence of an infection with a resistant strain.

Methods A patient was diagnosed with a recent HIV-1 infection in April 2005 and subsequently clinically monitored. HIV-1 evolution was studied by population sequencing of the first 984 bases of the *pol* gene as well as 454 ultra-deep pyrosequencing (UDPS) of parts of the *pol* and *env* genes.

Results The patient was diagnosed with a wild-type HIV-1 strain, but experienced rapid virological failure after initiating a non-nucleoside reverse transcriptase inhibitor (NNRTI)-based treatment regimen three years later. Population sequencing and UDPS revealed the presence of second HIV-1 strain with a Y188L NNRTI resistance mutation in a sample obtained shortly prior to initiation of therapy. Phylogenetic analyses showed that the two HIV-1 strains were genetically distinct, providing evidence for superinfection.

Conclusions The virological treatment failure in this patient was probably due to the superinfection with an NNRTI-resistant HIV-1 variant. Superinfection with drug resistant strains can undermine HIV-1 treatment regimens selected on the basis of resistance testing at diagnosis. Patients, especially in high-risk groups, as well as their clinicians, should be aware of the risks and dangers of superinfections.

Introduction

HIV-1 superinfection is defined as a second infection with a genetically distinct HIV-1 variant after the establishment of an adaptive immune response to the initial HIV-1 infection[1]. Estimates of the extent of superinfection differ widely between studies, ranging from no detected superinfections to an incidence comparable to that of initial infections[2, 3]. Superinfection may lead to failure of antiretroviral therapy because routine genotypic resistance testing at diagnosis may give misleading results. Superinfection with a wild-type virus can conceal an initial infection with a resistant variant[4-6], while superinfection with a resistant variant may go unnoticed after initial detection of a wild-type variant. The latter scenario was observed by Smith et al[7], who described a patient experiencing therapy failure after superinfection with an HIV-1 variant carrying two resistance mutations: the nucleoside reverse transcriptase inhibitor (NRTI)-related M184V and the protease inhibitor (PI)-related L90M. However, this patient was only intermittently compliant to his experimental triple NRTI regimen[7]. Here, we describe a recently infected HIV-1 patient who acquired a second, resistant variant that caused therapy failure. We confirm that the resistant variant was acquired through superinfection using ultra-deep pyrosequencing (UDPS).

Methods

Clinical monitoring

After diagnosis with a recent HIV-1 infection in April 2005, the patient was monitored by measurements of plasma HIV-1 RNA levels and $CD4^+$ T cell counts (Figure 1). Plasma samples were cryopreserved at -70ºC until sequencing analysis.

Viral sequencing

Routine genotypic resistance testing was performed using the ViroSeq assay (Abbott Molecular, Hoofddorp, the Netherlands), which is based on population sequencing of a region of the *pol* gene corresponding to protease and N-terminus of reverse transcriptase (GenBank accession numbers: JQ711496-JQ711501). This region was also subjected to UDPS as described elsewhere (454 Life Sciences, Branford, CT, USA)[8]. In short, three overlapping cDNA fragments were generated and used for the amplification of eight overlapping amplicons that were subjected to UPDS. Using this protocol, a consistent detection of low-level variants has been described[8, 9].

Another UDPS protocol was used to sequence one amplicon covering amino acids 254-375 of the *env* gene using a modification of a previously described *pol* UDPS protocol[10]. HIV RNA was extracted from plasma with the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) and used for RT-PCR with primers JA169-JA172 as previously described[11] with the following modifications: Thermoscript (Invitrogen,

Carlsbad, California, US) was used for reverse transcription and sample-specific sequence tags were added to the inner primers as described elsewhere[10]. After quality controls, amplicons from all samples were pooled and sequenced in both forward and reverse direction on the GS FLX System (Roche Applied Science) according to the manufacturer's instructions. The mean (±SD) efficiency of target RNA recovery of the *env* sequencing protocol is around 23% (20,9)[10].

Phylogenetic analysis

Population-based sequences were aligned with reference strains from the geographical area and the Los Alamos HIV database with ClustalW. A maximum likelihood tree was computed with MEGA 5.05 using the Tamura-Nei model with 1,000 bootstrap replicates.

The UDPS dataset was too large to compute a phylogenetic tree using all sequences; therefore two methods were used to analyze the dataset. First, all forward variants that were obtained with a frequency >1 and a length of >210 base pairs were analysed. Based on information obtained from the patient, his superinfection was most likely acquired in Germany. Therefore, sequences originating from Germany and the Netherlands were obtained from GenBank and the Los Alamos HIV database (Additional file 1). As the majority of these German and Dutch sequences represent subtype B, we have also included a subtype reference panel from the Los Alamos HIV database. All sequences were aligned to the HxB2 HIV reference sequence using ClustalX. A maximum likelihood tree was computed with MEGA 5.05 using the Hasegawa-Kishino-Yano model with 1000 bootstraps. A second analysis including all high-quality UDPS reads is described in Additional file 2.

Short tandem repeat (STR) profiling

Genomic DNA was extracted using the ChargeSwitch Forensic DNA purification kit (Invitrogen, Carlsbad, CA, USA). Subsequently, DNA samples were amplified with the AmpF/STR Identifier commercial DNA profiling kit and analysed using the ABI 3130xl capillary electrophoresis machine and Genemapper software v3.7 (Applied Biosystems). In all steps, standard manufacturers' protocols were followed. The European Network of Forensic Science Institutes DNA WG STR population database was used to calculate the likelihood that the samples originated from the same person.

Results

The patient reported having had repeated unprotected same-sex sexual encounters before and after HIV-1 diagnosis. After a negative HIV-antibody test in November 2004, he experienced flu-like symptoms in March 2005, suggesting an acute HIV-infection. Subsequently, he tested positive for HIV antibodies in

April 2005 with a plasma HIV-1 RNA level of 3.7 log^{10} copies/ml and a $CD4^+$ T cell count of 800 cells/mm^3 in June 2005. The patient was also diagnosed with genital herpes simplex virus type 2 (August 2007), hepatitis C virus (November 2007), urethral *Neisseria gonorrhoeae* (June 2005 and November 2007) and *Chlamydia trachomatis* (June 2005 and November 2007). In January 2007, 22 months after HIV-1 diagnosis, his plasma HIV RNA had increased to 4.7 log^{10} copies/ml and the CD4 count had dropped to 410 cells/mm^3. After two subsequent $CD4^+$ T cell counts below 350 cells/mm^3, therapy was initiated in March 2008. Based on a genotypic resistance test showing wild-type virus in June 2005, the patient was prescribed efavirenz, tenofovir and lamivudine. Although the patient reported good adherence to therapy, HIV-1 RNA levels remained high (Figure 1).

Additional genotypic resistance analyses were performed on samples from January 2007 and during therapy (Figure 1). Surprisingly, these analyses revealed a virus with the non-nucleoside reverse transcriptase inhibitor (NNRTI)-related resistance mutation Y188L in a different genetic background than the initial virus. Y188L confers high levels of resistance against the commonly used NNRTIs efavirenz and nevirapine[12]. Population sequencing of subsequent plasma samples revealed the appearance of two additional resistance mutations (K103N

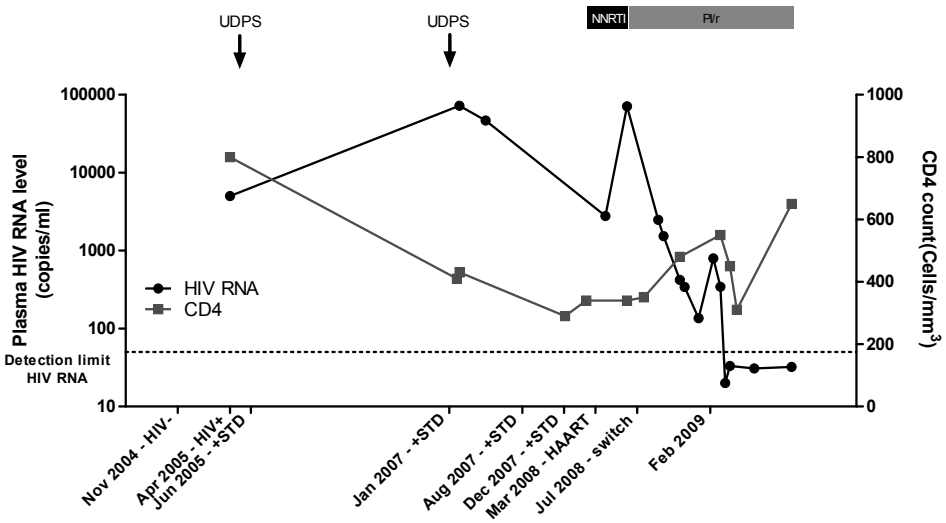


Figure 1. Clinical, virological and immunological follow-up of the patient. Clinical events such as diagnosis of sexually transmitted diseases (STDs) and initiation of HAART are shown on the X-axis. Therapy regimens are indicated in boxes above the graph. Non-nucleoside reverse transcriptase inhibitor (NNRTI) therapy consisted of efavirenz, tenofovir and lamivudine, and ritonavir-boosted protease therapy (PI/r) comprised ritonavir-boosted lopinavir, tenofovir and lamivudine. Arrows indicate the time points at which ultra-deep pyrosequencing (UDPS) was performed. -, negative; +, positive.

and M184V) in this second genetic background. After switching to a boosted PI-based regimen, an adequate viral response was obtained (Figure 1).

To confirm that the viral variant containing Y188L was different from the initial viral population, a maximum likelihood tree was computed based on all population sequences. This tree showed that the initial drug-susceptible virus was indeed phylogenetically unrelated to the patient's subsequent drug-resistant viral variants, suggesting a possible superinfection (Additional file 3).

To distinguish between co- and superinfection, UDPS was performed on a sample from June 2005 and a sample from January 2007 when the Y188L mutation was first detected (Figure 1). UDPS analysis of RT shortly after at diagnosis (June 2005) did not detect any viral variants with the Y188L mutation. Furthermore, several polymorphisms found in June 2005 could not be detected in the sample 22 months later (January 2007), and vice versa (Table 1). The same two samples were also subjected to UDPS of the V3 *env* region.

A phylogenetic tree based on all V3 variants detected as reads with a minimal frequency of two showed that sequences from the two time points formed two mutually exclusive, monophyletic clusters with bootstrap values of 86% and 88% (Figure 2). Similarity investigations and additional phylogenetic analyses (Additional file 4) corroborated the findings. Thus, the putative superinfecting strain was not detected in any of 60,591 *pol* and 14,085 *env* UDPS reads generated from the first plasma sample, which strongly indicates that it was not a minor variant in the initial inoculum.

Remarkably, the analyses also showed that the superinfecting resistant strain had completely replaced the initial population in plasma by January 2007, which indicates that it had much higher fitness. This fitness difference was not due to drug selective pressure as a complete replacement had occurred before HAART was initiated.

Table 1. Comparison of *Pol* Ultra-Deep pyrosequencing results at two time points.

		June 2005		January 2007	
	mutation	**variant %**	**reads**	**variant %**	**reads**
PR	L63P	100	10603	0	6291
	V71V	99.86	10582	0	6291
	V77I	0	10573	99.19	6305
	I93L	99.91	7790	0	5156
RT	Y188L	0	16118	98.76	6462

PR: Protease, RT: Reverse Transcriptase.
The average read length was 182.7 (June 2005) or 189.5 (January 2007) base pairs.

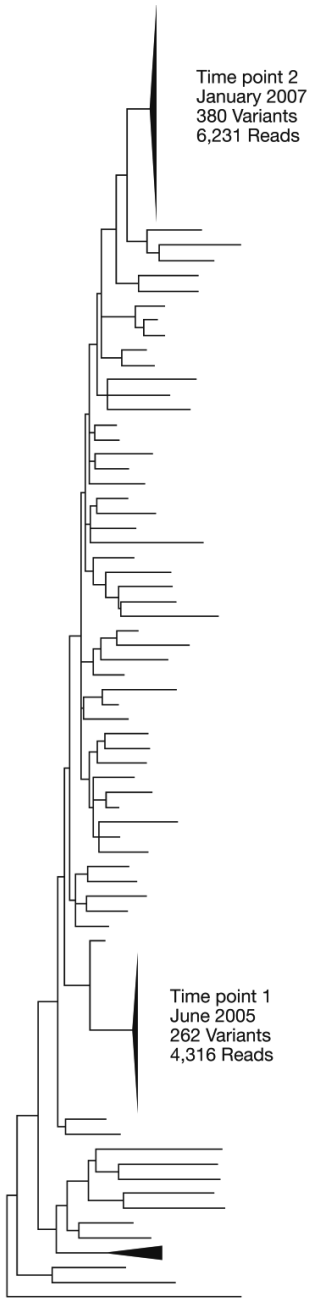


Figure 2. Maximum likelihood phylogenetic tree comparing *env* sequences obtained by ultra-deep pyrosequencing. A phylogenetic tree was calculated using all patient sequences obtained with a frequency of >1 and a length of >210 base pairs. Sequences from Los Alamos HIV database and GenBank were used as reference variants (Additional file 1). All clusters with a bootstrap value of ≥70 have been compressed.

To confirm that our samples belonged to the same patient, STR profiling was carried out on the sample of June 2005 and a sample from 2009 using methods typically applied in forensic investigations. A completely identical AmpF/STR Identifier profile was obtained from both samples providing strong support (likelihood ratio: $1.08x10^{14}$) that the two samples originated from the same individual (Additional file 5).

Discussion

We describe a patient who was diagnosed with a drug-sensitive virus, and three years later failed to respond to a NNRTI-based regimen due to superinfection with a NNRTI-resistant variant. To investigate the dynamics of the viral populations over time, we applied UDPS to study the *env* and *pol* sequences from two samples[8, 10]. We were able to show the absence of the resistant variants in a sample taken shortly after seroconversion by analysing 74,676 reads of 9 amplicons. Since no additional material of the initial time point was available, we were unable to confirm these findings using other approaches such as allele-specific PCR. The original wild-type variants were not detected in a sample obtained 2 years later, when all variants in the quasispecies harboured the Y188L NNRTI mutation. Based on these results we conclude that superinfection had occurred. This conclusion is corroborated by the continuous risk behaviour demonstrated by this patient as manifested by the acquisition of multiple sexually transmitted diseases. It remains unknown why the later variant was able to rapidly replace the initial virus population. As this switch occurred before therapy initiation, a fitness factor other than drug resistance must have been involved. Many viral and host factors have been described to influence the viral fitness, such as the presence of immune epitopes[13] or polymorphisms in other regions of the viral genome[14].

Smith et al.[7] were the first to describe a patient who acquired a second, resistant HIV-1 variant harbouring the NRTI-related M184V and PI-related L90M mutation. This patient also experienced therapy failure, but only after reported low adherence to an experimental triple NRTI regimen[7]. In our case the patient reported good adherence of his NNRTI-based regimen, providing a stronger link between superinfection and therapy failure.

The possibility of superinfection with a resistant variant has important clinical implications. Superinfection is a risk for HIV-positive individuals practising serosorting, i.e. unprotected sex with other HIV-1-infected persons. Patients, doctors and other health staff should be aware of the risk of superinfection with drug resistant HIV variants which may affect therapy efficacy. Clinicians may consider an (additional) genotypic analysis on a sample taken shortly before therapy initiation when their patient has been identified to be at high risk for superinfection.

Acknowledgements and funding

We thank Bozena Hanczaruk from 454 Life Sciences for excellent technical assistance.

This work was supported by the Aids Fonds, grant number 2007034.

References

1. Smith DM, Richman DD, Little SJ. HIV superinfection. *J Infect Dis* 2005; **192**: 438-44.
2. Smith DM, Wong JK, Hightower GK et al. Incidence of HIV superinfection following primary infection. *JAMA* 2004; **292**: 1177-8.
3. Rachinger A, Manyenga P, Burger JA et al. Low incidence of HIV-1 superinfection even after episodes of unsafe sexual behavior of homosexual men in the Amsterdam Cohort Studies on HIV Infection and AIDS. *J Infect Dis* 2011; **203**: 1621-8.
4. Koelsch KK, Smith DM, Little SJ et al. Clade B HIV-1 superinfection with wild-type virus after primary infection with drug-resistant clade B virus. *AIDS* 2003; **17**: F11-6.
5. Yang OO, Daar ES, Jamieson BD et al. Human immunodeficiency virus type 1 clade B superinfection: evidence for differential immune containment of distinct clade B strains. *J Virol* 2005; **79**: 860-8.
6. Brenner B, Routy JP, Quan Y et al. Persistence of multidrug-resistant HIV-1 in primary infection leading to superinfection. *AIDS* 2004; **18**: 1653-60.
7. Smith DM, Wong JK, Hightower GK et al. HIV drug resistance acquired through superinfection. *AIDS* 2005; **19**: 1251-6.
8. Simen BB, Simons JF, Hullsiek KH et al. Low-abundance drug-resistant viral variants in chronically HIV-infected, antiretroviral treatment-naive patients significantly impact treatment outcomes. *J Infect Dis* 2009; **199**: 693-701.
9. Le T, Chiarella J, Simen BB et al. Low-abundance HIV drug-resistant viral variants in treatment-experienced persons correlate with historical antiretroviral use. *PLoS One* 2009; **4**: e6079.
10. Mild M, Hedskog C, Jernberg J et al. Performance of ultra-deep pyrosequencing in analysis of HIV-1 pol gene variation. *PLoS One* 2011; **6**: e22741.
11. Leitner T, Korovina G, Marquina S et al. Molecular epidemiology and MT-2 cell tropism of Russian HIV type 1 variant. *AIDS Res Hum Retroviruses* 1996; **12**: 1595-603.
12. Bacheler L, Jeffrey S, Hanna G et al. Genotypic correlates of phenotypic resistance to efavirenz in virus isolates from patients failing nonnucleoside reverse transcriptase inhibitor therapy. *J Virol* 2001; **75**: 4999-5008.
13. Yang OO, Sarkis PT, Ali A et al. Determinant of HIV-1 mutational escape from cytotoxic T lymphocytes. *J Exp Med* 2003; **197**: 1365-75.
14. van der Kuyl AC, Kozaczynska K, Arien KK et al. Analysis of infectious virus clones from two HIV-1 superinfection cases suggests that the primary strains have lower fitness. *Retrovirology* 2010; **7**: 60.

Supplementary data

Additional File 1. Reference variants used for UDPS phylogenetic analysis

GenBank ID:

gi|190351973|gb|EU744073.1|
gi|190351975|gb|EU744074.1|
gi|1151159|gb|U43141.1|HIVU43141
gi|301323605|gb|GQ390599.1|
gi|301323607|gb|GQ390600.1|
gi|301323601|gb|GQ390597.1|
gi|19919757|gb|AF490512.1|
gi|62361768|gb|AY882421.1|
gi|227015892|gb|FJ895904.1|
gi|332138519|gb|JF278258.1|
gi|332138809|gb|JF278403.1|
gi|301323521|gb|GQ390557.1|
gi|301323555|gb|GQ390574.1|
gi|301323553|gb|GQ390573.1|
gi|301323557|gb|GQ390575.1|
gi|57472405|gb|AY878687.1|
gi|57472411|gb|AY878690.1|
gi|301323681|gb|GQ390637.1|
gi|301323683|gb|GQ390638.1|
gi|301323685|gb|GQ390639.1|
gi|57472415|gb|AY878692.1|
gi|57472413|gb|AY878691.1|
gi|156567719|gb|EF531336.1|
gi|190352169|gb|EU744171.1|
gi|190352171|gb|EU744172.1|
gi|190351775|gb|EU743974.1|
gi|190351777|gb|EU743975.1|
gi|301323685|gb|GQ390639.1|
gi|57472415|gb|AY878692.1|
gi|57472413|gb|AY878691.1|
gi|156567719|gb|EF531336.1|
gi|190352169|gb|EU744171.1|
gi|190352171|gb|EU744172.1|
gi|190351775|gb|EU743974.1|
gi|190351777|gb|EU743975.1|
gi|190351773|gb|EU743973.1|
gi|301323475|gb|GQ390534.1|
gi|301323473|gb|GQ390533.1|
gi|301323445|gb|GQ390519.1|
gi|328774854|gb|HQ386174.1|
gi|301641421|gb|GQ390680.1|
gi|301323721|gb|GQ390657.1|
gi|301641419|gb|GQ390679.1|
gi|301323717|gb|GQ390655.1|
gi|301323723|gb|GQ390658.1|
gi|301641415|gb|GQ390677.1|
gi|19919759|gb|AF490513.1|
gi|288189448|gb|GU191439.1|
gi|288189340|gb|GU191385.1|
gi|57472401|gb|AY878685.1|
gi|328774930|gb|HQ386212.1|
gi|301323841|gb|GQ390739.1|
gi|301323839|gb|GQ390738.1|
gi|301323837|gb|GQ390737.1|
gi|328774892|gb|HQ386193.1|
gi|156567721|gb|EF531337.1|
gi|328774786|gb|HQ386140.1|
gi|301323781|gb|GQ390709.1|
gi|301323785|gb|GQ390711.1|
gi|301323783|gb|GQ390710.1|
gi|301323869|gb|GQ390753.1|
gi|301323859|gb|GQ390748.1|
gi|301323861|gb|GQ390749.1|
gi|301323909|gb|GQ390773.1|
gi|301323875|gb|GQ390756.1|
gi|301323905|gb|GQ390771.1|
gi|301323817|gb|GQ390727.1|
gi|301323819|gb|GQ390728.1|
gi|301323813|gb|GQ390725.1|
gi|301323299|gb|GQ390446.1|
gi|301323309|gb|GQ390451.1|
gi|328774824|gb|HQ386159.1|
gi|301323367|gb|GQ390480.1|
gi|156567703|gb|EF531328.1|
gi|288189400|gb|GU191415.1|
gi|288189408|gb|GU191419.1|

gi|301323323|gb|GQ390458.1|
gi|115280025|gb|DQ871528.1|
gi|115279889|gb|DQ871460.1|
gi|301323343|gb|GQ390468.1|
gi|49617684|gb|AY669766.1|
gi|29409325|gb|AY093607.1|
gi|301323925|gb|GQ390781.1|
gi|301323945|gb|GQ390791.1|
gi|301323947|gb|GQ390792.1|
gi|282761896|gb|GU252765.1|
gi|282761910|gb|GU252772.1|
gi|190351973|gb|EU744073.1|
gi|190351975|gb|EU744074.1|
gi|1151159|gb|U43141.1|HIVU43141|

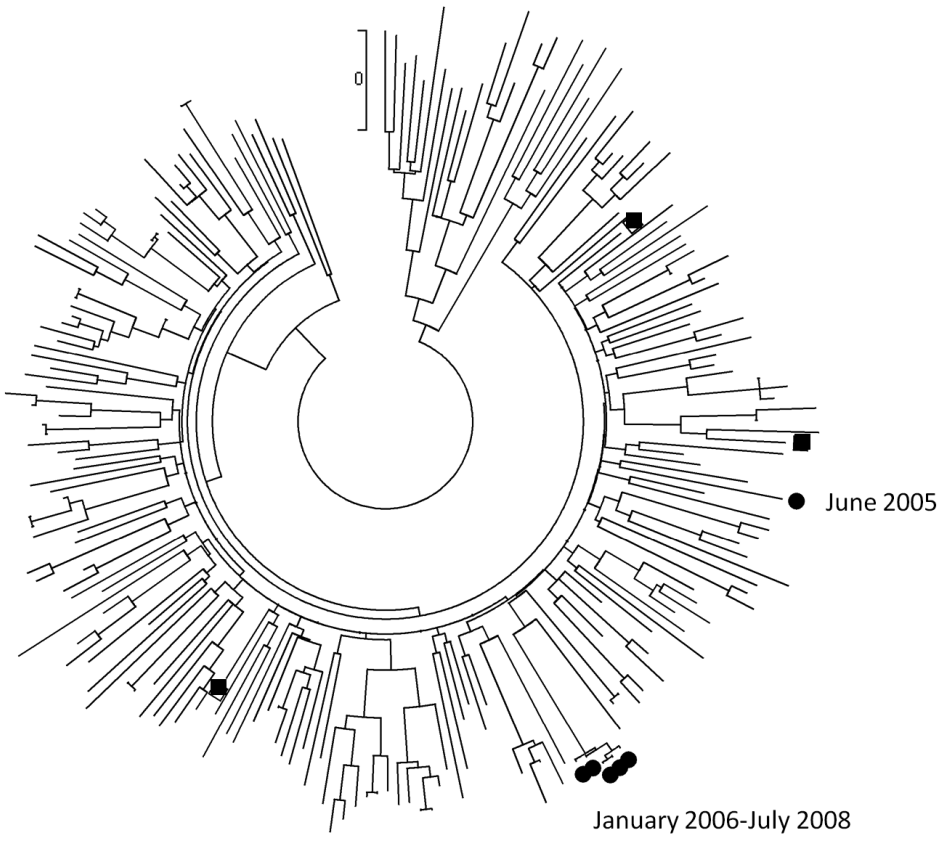
Los Alamos HIV database:

A.CD.97.97CD_KCC2.AM000053
03_AB.RU.97.KAL153_2.AF193276
B.DE.86.HAN.U43141
B.DE.86.D31.U43096
B.NL.00.671_00T103.AY423386
B.NL.86.H0320_2A12.U34603
B.NL.94.H1_46_5G2.EU744007
B.NL.95.H2_114_7H3.EU744052
B.NL.x.ENVVA.L08655
B.NL.90.H5_25_7G2.EU744159
B.NL.94.ACH142_*E11.DQ178989
B.NL.86.H434_8_A3.AY970946
B.NL.93.H3_40_10C9.EU744081
B.NL.x.168A.U15030
B.NL.96.H434_42_A1.AY970948
B.FR.83.HXB2_LAI_IIIB_BRU.K034
B.DE.86.HAN.U43141
B.FR.00.309_L_1.AY535455
B.BE.05.N2_WEEK_8.FJ653258
B.US.x.s35_d0_c23.EU604556
C.AR.01.ARG4006.AY563170
C.ZA.00.00ZAPCP1.AY529667
D.BR.96.patient_96BRRJ100.DQ14
D.CD.84.84ZR085.U88822
F2.CM.02.02CM_0016BBY.AY371158
F1.AO.06.AO_06_ANG125.FJ900269
G.BE.96.DRCBL.AF084936
B.NL.86.3202A21.U34604

Additional File 2. Description of supplementary analysis

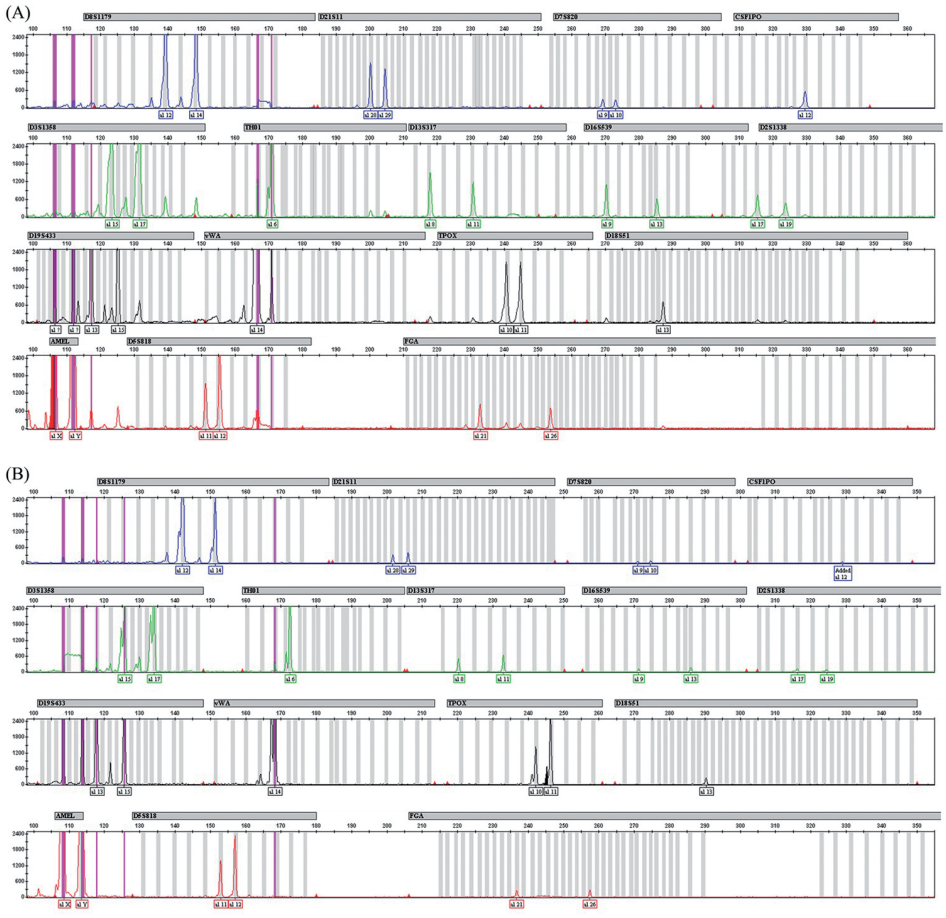
To make a more complete distinction between co- and superinfection, a second analysis of the UDPS results was performed using all *env* UDPS sequences. Pairwise global alignments of all reads of the June 2005 sample to the most common variant of the January 2007 sample were computed by the Needleman-Wunsch algorithm. A similar alignment of all reads of the January 2007 sample to the most common variant of the June 2005 was also calculated. This analysis was performed separately for all forward and reverse reads. All resulting alignments were used to calculate the similarity of all variants, revealing a maximum similarity of 93.0% between sample and reference variant while the minimum similarity of sequences of the same sample was 96.8%. An additional phylogenetic tree was constructed using twenty single reads longer than 210 bp with the lowest and highest similarity score of both samples. This again showed no intermingling of sequences.

Additional File 3. A supplementary figure displaying a phylogenetic tree of *pol* sequences of the patient. Maximum likelihood tree of all pol sequences of the patient and 206 local references. Circles indicate samples from the patient, squares of the samples handled at the same day as the initial sample of the patient.



Additional File 4. A supplementary figure displaying a phylogenetic tree of single *env* UDPS reads. Maximum likelihood tree of single, forward envelope reads of both time points (June 2005 and January 2007) with the lowest and highest similarity score as determined by the pairwise alignment.

Additional File 5. Comparison of short tandem repeat profiles of two samples. Panel A shows the short tandem repeat profile of the sample from June 2005, panel B from 2009.



Chapter 9

General Discussion

HIV variants harbouring transmitted drug resistance mutations (TDRM) can hamper effective treatment[1-5]. With a stable prevalence of about 10% in the Western world[6-8], transmission of drug resistant HIV variants remains an important issue. In this thesis, transmission and evolution of HIV harbouring TDRM variants was studied.

HIV Transmission

Sexual transmission is the main route of HIV infection[9]. During sexual transmission, HIV virions must overcome multiple hurdles before being able to establish an infection in a new host. The first-line of defence is the physiological barrier: the mucosa, low pH and epithelial layer[10, 11]. Second, numerous secreted factors in the genital mucosa have anti-HIV activity, such as defensins and type I interferons[12, 13]. Third, when HIV is able to enter a target cell, virions need to evade intracellular restriction factors, for example SAMHD1 for successful replication[13,14]. In addition, Langerhans cells (LCs), which are a subset of dendritic cells (DCs) residing in the epithelial layer of the genital tissue, can internalize and degrade virions. As such, LCs can prevent HIV transmission[15]. However, LCs may also facilitate HIV transmission. When the protective function of LCs is saturated in the presence of a high viral inoculum or down-regulated due to LC maturation, LCs may become infected and subsequently transmit HIV to $CD4^+$ T cells, the major target cells of HIV[16-18]. A second subset of DCs which can contribute to HIV transmission are DC-$SIGN^+$ DCs, which reside in the sub-epithelium of the genital tract[19]. Transmission by DCs may occur as a result of uptake and transfer of virions (in *trans*), or by infection and subsequent *de novo* virus production (in *cis*)[20, 21]. In addition, HIV transmission may occur independent of DCs by direct infection of $CD4^+$ T cells[22]. These extensively researched routes of HIV transmission are non-exclusive and the relative relevance *in vivo* remains undetermined (Schematic overview: Figure 1).

It is thought that during the first few days after transmission, HIV replication is limited to a small number of cells in the lymphatic tissue. Once a replication threshold is reached, HIV can disseminate systemicly[23, 24]. During these first few days, systemic dissemination may be prevented by factors decreasing replication such as aforementioned immune responses and post-exposure prophylaxis[22, 23]. In the majority of individuals, only one or few viral variants are disseminated, resulting in a very homogeneous quasispecies during early infection[25, 26].

We have investigated the impact of the replicative capacity (RC) of drug resistant HIV variants on the transmission efficacy. It has been described that some drug resistance mutations such as M184V in reverse transcriptase (RT) have a severe impact on the RC in primary T cells[27]. In this thesis, we demonstrated that HIV variants harbouring M184V are also less able to infect LCs and DC-$SIGN^+$ DCs, and

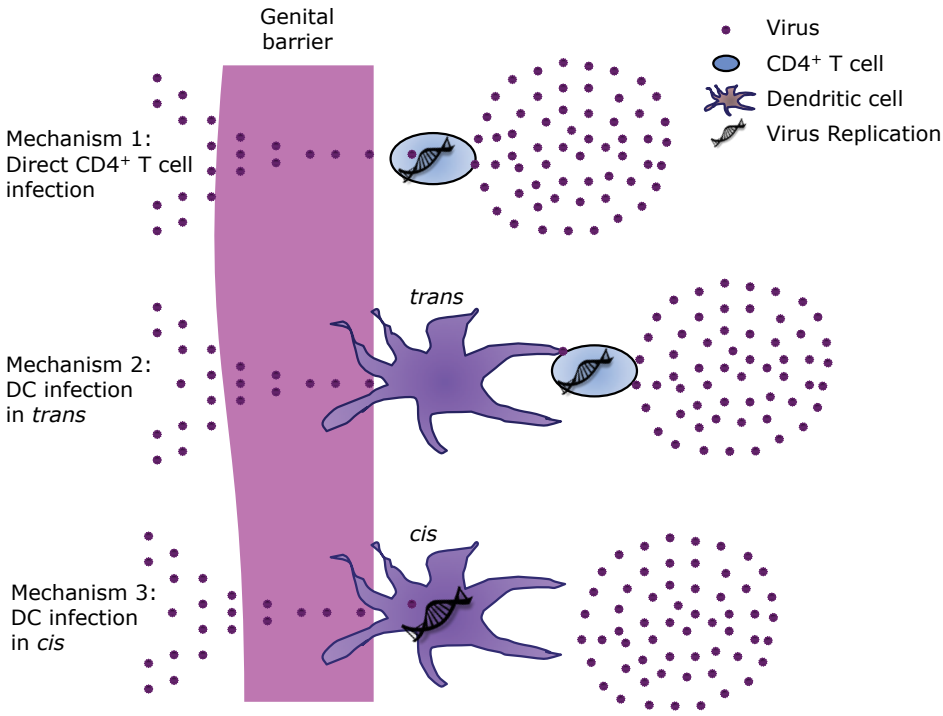


Figure 1. Biology of HIV transmission. Sexual transmission of HIV may occur by several non-exclusive routes. In mechanism 1, CD4+ T cells become infected directly. In mechanisms 2 and 3, dendritic cells such as langerhans cells and DC SIGN+ DCs are involved. DCs can take up and transfer HIV particles to CD4+ T cells in trans (mechanism 2) or become productively infected in cis (mechanism 3).

thereafter are less efficiently transmitted to target cells (Chapter 2). Therefore, the RC of HIV variants may be an important determinant of successful HIV transmission to the new host as it impacts multiple routes of HIV transmission. As a result, when a mixture of drug resistant and wild type virus is present within a source individual, preferred transmission of wild type virus with a higher RC may occur. On a population level, the chance of establishing an HIV infection per risk event may be smaller for HIV variants with a lower RC.

In the newly infected individual, it takes about ten days before HIV-RNA becomes detectable in plasma: the eclipse phase[28]. Subsequently, the transmitted virion(s) rapidly expand, reaching a peak viremea of 21-28 days post infection. At this time, HIV RNA levels are often above a million copies per ml, whereas $CD4^+$ T cells are rapidly depleted in the blood and especially in the gut-associated lymphoid tissue. The viral load gradually decreases while an adaptive immune response develops, reaching a stable viral set point three to six months after infection[29]. HIV specific

$CD8^+$ T cells and antibodies cause evolutionary pressure, leading to the selection of "escape variants", viral variants that are less recognized by the immune system[30].

Although the development of a natural immune response to HIV decreases viral replication, it is not protective against HIV infection. When an individual gains a second HIV infection with a genetically distinct HIV variant after the establishment of an adaptive immune response to the initial HIV infection it is called superinfection. Whereas large studies found a very low incidence of superinfection, detailed studies detected superinfection at a comparable rate to initial infections[31-33]. In Chapter 8, we described a patient who continued risk behaviour after HIV diagnosis and acquired a second HIV variant. This case is discussed in more detail below.

It has been extensively researched whether transmitted HIV variants have special characteristics. Detailed analysis of the quasispecies of source and index patient suggest a preferential transmission of ancestral strains rather than variants circulating in plasma of the source individual[34]. This implies that transmitted variants have an advantage in the HIV transmission bottleneck and that these transmitted variants remain present in the new host. Furthermore, transmitted HIV strains seem to have a distinct phenotype when it comes to the envelope. Envelopes of transmitted strains seem to contain shorter variable regions, unique glycosylation sites and binding potential[35-37]. These transmitted strains may remain present as minorities in plasma[38] or compartmentalize in the genital tract[39]. It would be interesting to investigate the evolution of *pol* of such persisting transmitted strains to gain more insight in transmission of drug resistance.

Evolution of HIV after Transmission

After transmission of HIV to a new host, drug resistance mutations selected in a previous host are no longer beneficial for the virus in the absence of drug pressure but may decrease the viral RC. In chapter 3, we identified three pathways describing evolution of HIV variants harbouring TDRM after transmission. First, reversion often occurs when resistance mutations have a high impact on RC, such as M184V in RT[40-42]. Second, atypical variants which are neither wild type or resistant variant may be selected when such atypical viral variants increase the RC. Atypical variants are frequently observed at positions where the difference between drug resistant and wild type amino acid is two nucleotides, such as position 215 in RT[43]. Third, persistence of TDRM is observed, which can be explained by two mechanisms. Persistence may occur when evolution is constrained by fixation through compensatory mutations, which has been demonstrated for the combination M41L and V60I in RT[44]. In addition, resistance mutations may persist when they have a minor impact on the RC[45]. In chapter 5,

we describe a group of patients infected with a circulating HIV variant harbouring a persisting mutational profile of four resistance-related mutations in RT. The RC of this viral variant was in the range of HIV wild type variants. This suggests that the TDRM are well-compensated resulting in either compensatory fixation or prolonged persistence due to limited RC advantage of revertants.

The fitness of a viral variant is not only determined by the RC but also by the host environment. In Chapter 8, we describe a patient who was initially infected with a drug susceptible HIV variant. Superinfection with a second drug resistant HIV variant resulted in therapy failure. Interestingly, the superinfecting drug resistant variant completely outcompeted the initial wild type HIV variant in this patient, indicating a higher fitness. Unfortunately, we did not have sufficient patient material to investigate viral and host factors explaining this outgrowth of a drug resistant virus.

As mentioned before, the immune system is an important force in HIV evolution. Although most immunological pressure is exerted on gag, env and nef[29, 46], epitopes in protease and RT have been described as well[47, 48]. For some resistance mutations located on epitopes it has been described that the peptide including drug resistance mutation can elicit both stronger or weaker responses from cytotoxic T cells than epitopes with wild type amino acids[48, 49]. In that case, the immune response can drive evolution towards persistence or reversion of resistance mutations. However, lists for the investigation of transmitted drug resistance are designed to only include mutations selected by exposure to drugs in a previous host[50, 51]. Thus, it is unlikely that included drug resistance mutations are selected by the immune system rather than exposure to drugs in a previous host.

Sources of Drug Resistant HIV Variants

In 75-80% of treatment-experienced patients with a detectable plasma HIV RNA, viral variants with reduced drug susceptibility can be detected, this often concerns resistance against several drug classes[52, 53]. These patients are an obvious source of transmitted drug-resistant HIV variants. However, onward transmission of variants harbouring TDRM by treatment-naïve patients also plays a major role in the epidemiology of transmitted drug resistance.

The epidemiology of transmitted drug resistance provides important insights in the sources of HIV variants carrying TDRM. In contrast to therapy-experienced patients, HIV variants in newly diagnosed patients mostly harbour resistance profiles limited to single drug classes[54]. Mostly this concerns thymidine analogue mutations (TAMs), especially M41L and variants at position 215[8, 55]. TAMs (M41L, D67N, K70R, L210W, T215Y/F, K219E/Q) are selected by zidovudine and

stavudine, two nucleoside reverse transcriptase inhibitors (NRTIs) that have been extensively used as antiretroviral treatment[56-58]. Current therapy regimens are increasingly successful in suppressing HIV RNA, resulting in a lower incidence of selecting drug resistance[55, 59, 60] and rarely select TAMs[61, 62]. Although, after transmission certain mutations may revert in the absence of drugs, limited TAM profiles tend to persist over time (chapter 3 and 4). Thus, a large proportion of transmitted drug resistance mutations that are observed nowadays were most likely selected in the past and are the result of onward transmission.

In addition, phylogenetic clustering of transmitted HIV variants strongly suggests onward transmission as several groups have described transmission clusters of recently infected or newly diagnosed patients[63-65]. As described in chapter 5, we also observed a transmission cluster containing viruses carrying a stable profile of TDRM.

Furthermore, novel, very sensitive sequencing methods can provide a more comprehensive view of the viral quasispecies, providing insights in the transmitted viral variants. In chapter 6 we investigated the presence of clinically relevant minority variants by deep sequencing of baseline samples and virological follow-up after therapy initiation of patients diagnosed with HIV variants harbouring a single TDRM. The absence of minority variants in these patients suggests onward transmission of variants with TDRM rather than transmission from therapy-experienced sources. However, we cannot completely exclude reversion as mutations will eventually revert below the detection limit of sensitive methods as well[66].

The long-term persistence and subsequent onward transmission of HIV variants harbouring TDRM imply that these variants are establishing a stable role in the HIV epidemic. It is important to determine the impact of these circulating HIV variants on drugs currently used in clinical practice, and for antiretrovirals used as prevention of transmission.

Implications of Transmitted Drug Resistance

Clinical Impact of Frequently Transmitted HIV Variants Containing TDRM

Current recommended first-line regimens contain two NRTIs combined with either a non-nucleoside reverse transcriptase inhibitor (NNRTI), boosted protease inhibitors (PIs), or integrase inhibitor[67, 68]. Large studies have demonstrated that transmitted drug resistant HIV variants can contribute to virological therapy failure[1-5]. However, such studies often investigate transmitted variants harbouring TDRM in general, rather than specific resistance profiles.

In patients diagnosed with a HIV variant carrying TDRM, the most frequently observed resistance mutations are the NRTI-related mutations M41L and

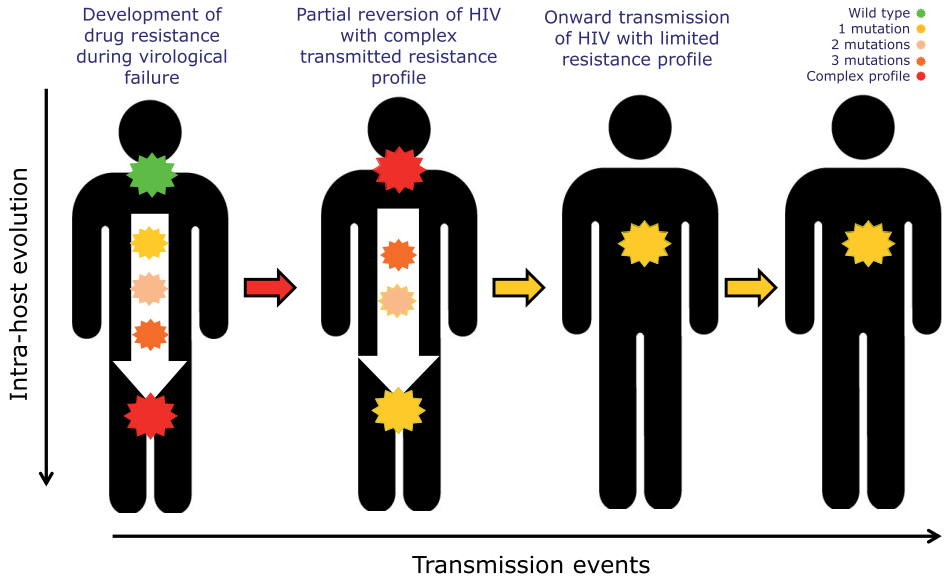


Figure 2. Onward transmission of HIV. The Y-axis represents evolution within the host, the arrows depict 3 transmission events between 4 hosts. The left individual was infected with a drug susceptible virus (green), but drug resistance mutations accumulate during treatment failure. The second individual is infected with an HIV variant harbouring a complex drug resistance profile (red), which partially reverts in the absence of drugs. This partially reverted virus (yellow) persists and is subsequently transmitted to two new hosts.

variants at position 215, and the NNRTI-related K130N[7, 8]. M41L and T215Y/F are both TAMs selected by drugs extensively used in the past, but which can contribute to broad cross-resistance against currently used drugs. At position 215, the selection of resistant amino acids tyrosine or phenylalanine requires two nucleotide changes from the wild type amino acid threonine. After transmission of variants harbouring tyrosine or phenylalanine at position 215, single mutations resulting in intermediate or atypical codons are often observed. It has been described that such intermediate and atypical variants at position 215 do not cause decreased susceptibility against thymidine analogues. However, the presence of a 215 variant in a transmitted virus decreases the genetic barrier for zidovudine[69] and increases the risk of therapy failure after initiation of a first-line regimen containing thymidine analogues[70]. It remained an important question whether limited TAM profiles impact the efficacy of current first-line regimens. We demonstrated that the presence of a single M41L has no impact on selection of drug resistance by tenofovir (TDF) and emtricitabine (FTC) *in vivo* or *in vitro* (chapter 7). As a result, initiation of a high genetic barrier regimen with a higher pill burden, increased toxicity and elevated costs may not be necessary when a

single M41L is detected at baseline. Indeed, a retrospective study investigating the impact of a single TAM in transmitted HIV variants revealed no difference on the virological outcome of current first-line regimens (C. van Nispen tot Pannerden, personal communication).

This limited impact for TAMs is in sharp contrast to the NNRTI-related K103N. The presence of K103N renders HIV completely resistant against the frequently used NNRTIs efavirenz and nevirapine[71]. As a result, transmission of viral variants harbouring K103N has a high impact on treatment efficacy[2].

Expanding the Investigation of Transmitted Drug Resistant HIV Variants

A viral variant must be present in the quasispecies as at least 10-25% of the viral population if it is to be detected by standard population sequencing based assays. Due to reversion of transmitted resistance mutations, population sequencing may not always be able to detect transmitted drug resistant HIV variants. However, resistance mutations can remain present as minority variants in plasma, archived as proviral DNA or in compartments other than blood. The presence of minority variants harbouring additional TDRM may contribute to therapy failure[72]. Technical advances have made it possible to investigate the viral population in more detail, for example by single-genome sequencing[73], allele-specific PCR[74] and ultra-deep pyrosequencing[75]. We used the state-of-the-art technique ultra-deep pyrosequencing to investigate the presence of additional resistant minority variants in patients diagnosed with an HIV variant harbouring a single TDRM. As described in Chapter 6, such resistant minority variants could be detected in only one out of ten patients. Larger studies are warranted to investigate the possible association between the mutational profile observed by population sequencing and the presence of additional drug resistant minority variants.

Current diagnostic resistance tests usually only determine resistance mutations in protease and the N-terminus of RT. However, outside of this region resistance mutations related to protease and RT inhibitors can also be selected.

Mutations in gag, the natural substrate of protease, are involved in the development of resistance against protease inhibitors[76-78]. The limited research on natural variation in gag makes it difficult to distinguish between polymorphisms and drug resistance mutations. This complicates the determination of the impact of gag mutations on virological failure to PIs[79] and the investigation of transmitted drug resistance in gag.

There is mounting evidence that mutations in the connection and RNaseH domain of RT contribute to drug resistance against NRTIs and compensation of a decreased RC caused by resistance mutations[80]. Inclusion of gag and the

C-terminus of RT in genotypic analysis and epidemiological studies may provide useful insights in the clinical relevance of these mutations and on transmission of drug resistant HIV variants.

HIV treatment has greatly improved in recent years, resulting in increased virological suppression and decreased selection of drug resistance[55, 59]. However, a number of individuals still experience therapy failure, possibly related to selection or transmission of drug resistance. Fortunately, an increasing number of drugs is available for the treatment of HIV. The list of recommended first-line choices NRTIs, NNRTIs and PIs has recently been expanded to include the integrase inhibitor raltegravir[68, 81]. In addition, the fusion inhibitor enfuvirtide (T-20), and co-receptor antagonist maraviroc have been approved for clinical use[82]. In total, 24 drugs are currently available for HIV treatment, and more are on their way. Although therapy regimens have improved, drug resistant HIV-variants can still be selected and subsequently transmitted. After approval of enfuvirtide and raltegravir it took three and four years respectively until transmission of drug resistance against these new drug classes was described[83-85]. If the use of drugs from additional drug classes increases, it is also likely that transmission of HIV variants harbouring TDRM against these drug classes increases. In that case, monitoring of transmitted HIV-variants harbouring TDRM against such new drugs becomes important. In addition, molecular epidemiology including genes affected by newly approved drug classes may provide insights in the sources of transmitted drug resistant variants and the transmission dynamics of HIV.

Prevention of Transmission with Antiretroviral Therapy

Although huge efforts are made to decrease transmission of HIV and new infections have decreased 15% in the last 10 years, an estimated 6.000 people are newly infected worldwide every day[86]. One of the strategies to prevent HIV transmission is pre-exposure prophylaxis (PrEP), the treatment of uninfected individuals to reduce the risk of HIV infection. After promising results in primate models[87-90], several large studies in different populations and risk groups have been performed with oral or gel formulated tenofovir (TDF) or tenofovir/emtricitabine (TFD/FTC)[91-95]. Whereas some studies were discontinued due to lack of efficacy[93, 96], the majority of studies described an incidence reduction of 39 to 75%, and even higher when incompliant participants were censored. The U.S. food and drug administration (FDA) recently approved the use of TDF/FTC as prevention for HIV uninfected individuals with high risk for HIV infection. However, there are several downsides of TDF/FTC as PrEP. The costs for antiretrovirals remain high, drugs need to be taken daily and toxicity can be a problem. In addition, development of drug resistance is a major concern[97]. It has been described that HIV drug resistance is selected in some individuals infected during PrEP, although a case-report described low-level viremia with wild

type virus and delayed seroconversion[98]. To prevent selection of drug resistance, HIV tests are recommended at least every three months during PrEP. Modelling predicted that when risk behavior remains stable, the absolute transmission of wild type and drug resistant HIV variants will decrease[99].

The impact of transmitted drug resistance on the efficacy of PrEP is thought to be small, as variants resistant to both TDF and FTC are rarely observed in newly diagnosed patients[97]. However, it remained unknown whether the frequently transmitted M41L-HIV variants have an impact on efficacy of PrEP, or development of resistance during PrEP. In Chapter 8, we demonstrated that the presence of M41L does not affect the susceptibility of HIV to TDF and FTC, nor change the selected resistance profile. However, as both drugs in PrEP are frequently used in clinical practice, transmission from patients experiencing therapy failure is still a risk. PrEP strategies may be improved by using drugs for PrEP that are not commonly used as treatment, limiting the clinical consequences when drug resistance does develop. Furthermore, new drugs with a very long half live are being developed[100] which may decrease pill burden and thus increase adherence. However, such long-lived drugs also provide new challenges in preventing the development of drug resistance as their activity cannot be stopped if an individual becomes infected despite PrEP.

A second strategy that is currently explored for prevention of HIV transmission is "Test and treat". As there is a strong correlation between plasma HIV RNA and risk of HIV transmission[101], it was hypothesized that HIV treatment may prevent HIV transmission. Indeed, large studies have shown a reduction in HIV transmission of up to 96% in discordant couples of whom the HIV positive partner achieved an undetectable HIV RNA due to treatment[102, 103]. Vertical transmission from HIV positive mothers to their babies is very common (15-45%) but can be greatly reduced with drug interventions, and decreases to <0.5% when mothers have an HIV RNA below 50 copies/ml[104-106]. Mathematical modelling showed that HIV transmission can be reduced or even eliminated when everyone at risk for HIV infection is tested yearly and treated immediately after diagnosis[107, 108]. However, in daily practice many challenges need to be overcome to optimize the impact of "test and treat" for effective prevention. Development and transmission of drug resistance may increase if "test and treat" is implemented, compromising the positive effects.

Transmission of Drug Resistant Viruses

Almost thirty years of extensive HIV research has not only greatly improved our knowledge of HIV and the prospects for patients, it also pushed forward the knowledge on immunology and viral evolution. Insights gained in the field of HIV may be applicable to other viruses, and vice versa.

Influenza, the causative agent of flu, causes severe symptoms in three to five million people worldwide, killing about 250,000 to 500,000 yearly[109]. Two classes of drugs are available to prevent and treat influenza virus: M2 ion channel inhibitors and neuraminidase inhibitors. Resistance to oseltamivir, the most important neuraminidase inhibitor, is caused by a single mutation in neuraminidase.

The 2009 pandemic strain was resistant against the M2 ion channel inhibitor amantadine but the prevalence of H275Y remained low and almost exclusively occurred in patients who had been treated with oseltamivir[110, 111]. However, transmission of pandemic influenza with H275Y has been described on a small scale[112]. Animal experiments revealed that pandemic influenza resistant to oseltamivir is efficiently transmitted[113]. The risk of large scale transmission of mantadine and oseltamivir resistant influenza therefore remains a possible scenario.

Although a single H275Y comes with a fitness cost *in vitro*, compensatory mutations in neuraminidase may cause a higher transmissibility of resistant influenza[114]. As the balance between neuraminidase and hemagglutinin is essential for influenza, compensatory mutations may also be selected in hemagglutinin. When more compounds become available for treatment of influenza, combination therapy may be useful to hamper or even prevent selection of drug resistance.

This rapid selection and transmission of drug resistant influenza variants is in sharp contrast to herpes viruses. Herpes simplex virus (HSV) has a very high prevalence and individuals remain infected for the rest of their lives. During latency there is no viral production. During primary and recurrent infections however, even when asymptomatic, virus production, shedding, and possible transmission occurs[115]. The nucleoside analogue acyclovir is used on a large scale for treatment, suppression and prophylaxis of herpes viruses. Interestingly, the prevalence of acyclovir-resistant HSV is several times higher in immune-compromised patients than in immune-competent patients[116, 117]. This is probably due to higher exposure to drugs and increased replication in immune-compromised patients. Drug resistance is usually selected in the viral thymidine kinase TK, which is necessary to phosphorylate and thereby activate acyclovir[118]. However, a mouse study showed that a functional TK increases the virulence and capacity to reactivate of HSV[119], which may contribute to the low rate of selection and transmission of drug resistant HSV.

Treatment of HSV with acyclovir inhibits HIV replication in co-infected patients. *In vitro*, the propagation of HIV under pressure of acyclovir results in selection of the NRTI-related resistance mutations V75I and M184I[120]. As these mutations impact the sensitivity of HIV to NRTIs[71, 121], this needs to be considered when treating HSV and HIV co-infected patients.

Prospects of Transmission of Drug Resistant HIV Variants

Combination antiretroviral therapy has changed the future of HIV infected individuals. Instead of merely delaying a fatal disease by drugs that unavoidably resulted in selection of drug resistant HIV variants, modern treatment options have changed HIV into a chronic infection. Current therapy regimens contain fewer pills, cause less toxicity and select for drug resistance less frequently. However, the efficacy of these regimens can be hampered by transmission of drug resistant HIV variants. Especially the increasing prevalence of transmitted NNRTI-resistance in newly diagnosed patients during recent years is worrying[7, 122], as only a single mutation is required for high levels of resistance against the first generation NNRTI. Well tolerable, potent new drugs, preferably complete regimens combined in a single tablet, will be beneficial to treat patients with transmitted as well as acquired drug resistant HIV variants.

Suppression of the viral load does not only improve the outcome of the patient on therapy, it also greatly reduces the chance of HIV transmission[102, 103]. In addition, PrEP may contribute to prevention of transmission. However, prevention of transmission through the use of the same antiretrovirals as those used to treat infected individuals does carry the risk of selection and circulation of HIV variants harbouring TDRM. Therefore, development of user friendly and potent novel therapeutics with a different mode of action to prevent HIV transmission will hopefully succeed in the near future. To achieve this, it is important to further unravel the biology of HIV transmission *in vivo.*

In the past decade, huge efforts have been made to increase treatment in resource-limited settings, where the disease burden is highest. Although availability of treatment increases life expectancy and quality of many individuals and decreases the chance of HIV transmission[103, 123], it also leads to selection and transmission of drug resistant HIV variants[124, 125]. To minimize the problems of selection and the subsequent transmission of drug resistant HIV variants, it is essential to provide affordable monitoring of therapy efficacy and drug resistance testing. Furthermore, in resource-limited settings, subtypes other than the most studied subtype B predominate. Selection of drug resistance can differ for non-B viruses[126, 127]. It is of great importance to elucidate the differences in selection of drug resistance for non-B subtypes, both for wild type strains and for frequently observed variants harbouring TDRM.

Conclusions

Drug resistant HIV variants with a diminished RC also have a lower transmission efficacy. In case drug resistant HIV variants are transmitted, reversion of mutations can occur in the absence of drugs. However, mutations can also persist due to a low impact on RC or the selection of compensatory mutations. Onward transmission of established HIV variants harbouring TDRM is a major force in fuelling the epidemic of drug resistant HIV variants. Such stable, frequently transmitted variants which were mostly selected in the past may have limited clinical impact for current first-line regimens. Further epidemiological, clinical and virological research studying transmission of drug resistant HIV remains warranted.

References

1. Wittkop L, Gunthard HF, de Wolf F et al. Effect of transmitted drug resistance on virological and immunological response to initial combination antiretroviral therapy for HIV (EuroCoord-CHAIN joint project): a European multicohort study. *Lancet Infect Dis* 2011; **11**: 363-71.
2. Kuritzkes DR, Lalama CM, Ribaudo HJ et al. Preexisting resistance to nonnucleoside reverse-transcriptase inhibitors predicts virologic failure of an efavirenz-based regimen in treatment-naive HIV-1-infected subjects. *J Infect Dis* 2008; **197**: 867-70.
3. DeGruttola V, Dix L, D'Aquila R et al. The relation between baseline HIV drug resistance and response to antiretroviral therapy: re-analysis of retrospective and prospective studies using a standardized data analysis plan. *Antivir Ther* 2000; **5**: 41-8.
4. Chaix ML, Desquilbet L, Descamps D et al. Response to HAART in French patients with resistant HIV-1 treated at primary infection: ANRS Resistance Network. *Antivir Ther* 2007; **12**: 1305-10.
5. Bansi L, Geretti AM, Dunn D et al. Impact of transmitted drug-resistance on treatment selection and outcome of first-line Highly Active Antiretroviral Therapy (HAART). *J Acquir Immune Defic Syndr* 2010; **53**: 633-9.
6. Wensing AM, van de Vijver DA, Angarano G et al. Prevalence of drug-resistant HIV-1 variants in untreated individuals in Europe: implications for clinical management. *J Infect Dis* 2005; **192**: 958-66.
7. SPREAD. Recent dynamics of transmitted drug resistance in Europe: SPREAD programme 2006-2007. *9th European Workshop on HIV & Hepatitis Treatment Strategies & Antiviral Drug Resistance*. Paphos, Cyprus: Reviews in Antiviral Therapy & Infectious Diseases, 2011; 15-6.
8. Wheeler WH, Ziebell RA, Zabina H et al. Prevalence of transmitted drug resistance associated mutations and HIV-1 subtypes in new HIV-1 diagnoses, U.S.-2006. *AIDS* 2010; **24**: 1203-12.
9. UNAIDS. Together we will end AIDS. 2012.
10. Lai SK, Hida K, Shukair S et al. Human immunodeficiency virus type 1 is trapped by acidic but not by neutralized human cervicovaginal mucus. *J Virol* 2009; **83**: 11196-200.
11. Shukair SA, Allen SA, Cianci GC et al. Human cervicovaginal mucus contains an activity that hinders HIV-1 movement. *Mucosal Immunol* 2012.
12. Borrow P, Shattock RJ, Vyakarnam A. Innate immunity against HIV: a priority target for HIV prevention research. *Retrovirology* 2010; **7**: 84.

13. Carrington M, Alter G. Innate Immune Control of HIV. *Cold Spring Harb Perspect Med* 2012; **2**: a007070.
14. Ayinde D, Casartelli N, Schwartz O. Restricting HIV the SAMHD1 way: through nucleotide starvation. *Nat Rev Microbiol* 2012; **10**: 675-80.
15. de Witte L, Nabatov A, Pion M et al. Langerin is a natural barrier to HIV-1 transmission by Langerhans cells. *Nat Med* 2007; **13**: 367-71.
16. Spira AI, Marx PA, Patterson BK et al. Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques. *J Exp Med* 1996; **183**: 215-25.
17. Hu J, Gardner MB, Miller CJ. Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. *J Virol* 2000; **74**: 6087-95.
18. Fahrbach KM, Barry SM, Anderson MR et al. Enhanced cellular responses and environmental sampling within inner foreskin explants: implications for the foreskin's role in HIV transmission. *Mucosal Immunol* 2010; **3**: 410-8.
19. Geijtenbeek TB, Kwon DS, Torensma R et al. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* 2000; **100**: 587-97.
20. Turville SG, Santos JJ, Frank I et al. Immunodeficiency virus uptake, turnover, and 2-phase transfer in human dendritic cells. *Blood* 2004; **103**: 2170-9.
21. de Witte L, Nabatov A, Geijtenbeek TB. Distinct roles for DC-SIGN+-dendritic cells and Langerhans cells in HIV-1 transmission. *Trends Mol Med* 2008; **14**: 12-9.
22. Miller CJ, Shattock RJ. Target cells in vaginal HIV transmission. *Microbes Infect* 2003; **5**: 59-67.
23. Miller CJ, Li Q, Abel K et al. Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. *J Virol* 2005; **79**: 9217-27.
24. Zhang Z, Schuler T, Zupancic M et al. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science* 1999; **286**: 1353-7.
25. Keele BF, Giorgi EE, Salazar-Gonzalez JF et al. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A* 2008; **105**: 7552-7.
26. Gottlieb GS, Heath L, Nickle DC et al. HIV-1 variation before seroconversion in men who have sex with men: analysis of acute/early HIV infection in the multicenter AIDS cohort study. *J Infect Dis* 2008; **197**: 1011-5.
27. Back NK, Nijhuis M, Keulen W et al. Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. *EMBO J* 1996; **15**: 4040-9.
28. Fiebig EW, Wright DJ, Rawal BD et al. Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. *AIDS* 2003; **17**: 1871-9.
29. McMichael AJ, Borrow P, Tomaras GD et al. The immune response during acute HIV-1 infection: clues for vaccine development. *Nat Rev Immunol* 2010; **10**: 11-23.
30. Goonetilleke N, Liu MK, Salazar-Gonzalez JF et al. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med* 2009; **206**: 1253-72.
31. Redd AD, Mullis CE, Serwadda D et al. The rates of HIV superinfection and primary HIV incidence in a general population in Rakai, Uganda. *J Infect Dis* 2012; **206**: 267-74.
32. Rachinger A, Manyenga P, Burger JA et al. Low incidence of HIV-1 superinfection even after episodes of unsafe sexual behavior of homosexual men in the Amsterdam Cohort Studies on HIV Infection and AIDS. *J Infect Dis* 2011; **203**: 1621-8.

33. Smith DM, Wong JK, Hightower GK et al. Incidence of HIV superinfection following primary infection. *JAMA* 2004; **292**: 1177-8.
34. Redd AD, Collinson-Streng AN, Chatziandreou N et al. Previously Transmitted HIV-1 Strains Are Preferentially Selected During Subsequent Sexual Transmissions. *J Infect Dis* 2012.
35. Chohan B, Lang D, Sagar M et al. Selection for human immunodeficiency virus type 1 envelope glycosylation variants with shorter V1-V2 loop sequences occurs during transmission of certain genetic subtypes and may impact viral RNA levels. *J Virol* 2005; **79**: 6528-31.
36. Sagar M, Laeyendecker O, Lee S et al. Selection of HIV variants with signature genotypic characteristics during heterosexual transmission. *J Infect Dis* 2009; **199**: 580-9.
37. Nawaz F, Cicala C, Van Ryk D et al. The genotype of early-transmitting HIV gp120s promotes alpha (4) beta(7)-reactivity, revealing alpha (4) beta(7) +/CD4+ T cells as key targets in mucosal transmission. *PLoS Pathog* 2011; **7**: e1001301.
38. Love TM, Thurston SW, Keefer MC et al. Mathematical modeling of ultradeep sequencing data reveals that acute CD8+ T-lymphocyte responses exert strong selective pressure in simian immunodeficiency virus-infected macaques but still fail to clear founder epitope sequences. *J Virol* 2010; **84**: 5802-14.
39. Whitney JB, Hraber PT, Luedemann C et al. Genital tract sequestration of SIV following acute infection. *PLoS Pathog* 2011; **7**: e1001293.
40. Jain V, Sucupira MC, Bacchetti P et al. Differential persistence of transmitted HIV-1 drug resistance mutation classes. *J Infect Dis* 2011; **203**: 1174-81.
41. Pingen M, Nijhuis M, de Bruijn JA et al. Evolutionary pathways of transmitted drug-resistant HIV-1. *J Antimicrob Chemother* 2011; **66**: 1467-80.
42. Wainberg MA, Moisi D, Oliveira M et al. Transmission dynamics of the M184V drug resistance mutation in primary HIV infection. *J Antimicrob Chemother* 2011; **66**: 2346-9.
43. de Ronde A, van Dooren M, van Der Hoek L et al. Establishment of new transmissible and drug-sensitive human immunodeficiency virus type 1 wild types due to transmission of nucleoside analogue-resistant virus. *J Virol* 2001; **75**: 595-602.
44. MCDG H, Albert J, Lindstrom A et al. Compensatory Fixation Explains Long Term Persistence of the M41L in HIV-1 Reverse Transcriptase in a Large Transmission Cluster. *15th International HIV Drug Resistance Workshop: Basic Principles and Clinical Implications*. Sitges, Spain: Antiviral Therapy, 2006; S113.
45. Xu HT, Oliveira M, Quan Y et al. Differential impact of the HIV-1 non-nucleoside reverse transcriptase inhibitor mutations K103N and M230L on viral replication and enzyme function. *J Antimicrob Chemother* 2010; **65**: 2291-9.
46. Brumme ZL, Brumme CJ, Carlson J et al. Marked epitope- and allele-specific differences in rates of mutation in human immunodeficiency type 1 (HIV-1) Gag, Pol, and Nef cytotoxic T-lymphocyte epitopes in acute/early HIV-1 infection. *J Virol* 2008; **82**: 9216-27.
47. HIV Molecular Immunology Database. http://www.hiv.lanl.gov/content/immunology/index.html.
48. Mason RD, Bowmer MI, Howley CM et al. Antiretroviral drug resistance mutations sustain or enhance CTL recognition of common HIV-1 Pol epitopes. *J Immunol* 2004; **172**: 7212-9.
49. Mueller SM, Spriewald BM, Bergmann S et al. Influence of major HIV-1 protease inhibitor resistance mutations on CTL recognition. *J Acquir Immune Defic Syndr* 2011; **56**: 109-17.
50. Bennett DE, Camacho RJ, Otelea D et al. Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. *PLoS One* 2009; **4**: e4724.

51. Attia S, Egger M, Muller M et al. Sexual transmission of HIV according to viral load and antiretroviral therapy: systematic review and meta-analysis. *AIDS* 2009; **23**: 1397-404.
52. Richman DD, Morton SC, Wrin T et al. The prevalence of antiretroviral drug resistance in the United States. *AIDS* 2004; **18**: 1393-401.
53. van de Vijver DA, Wensing AM, Asjo B et al. HIV-1 drug-resistance patterns among patients on failing treatment in a large number of European countries. *Acta Dermatovenerol Alp Panonica Adriat* 2010; **19**: 3-9.
54. Transmission of drug-resistant HIV-1 in Europe remains limited to single classes. *AIDS* 2008; **22**: 625-35.
55. Vercauteren J, Deforche K, Theys K et al. The incidence of multidrug and full class resistance in HIV-1 infected patients is decreasing over time (2001-2006) in Portugal. *Retrovirology* 2008; **5**: 12.
56. Boucher CA, O'Sullivan E, Mulder JW et al. Ordered appearance of zidovudine resistance mutations during treatment of 18 human immunodeficiency virus-positive subjects. *J Infect Dis* 1992; **165**: 105-10.
57. Larder BA, Darby G, Richman DD. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. *Science* 1989; **243**: 1731-4.
58. Larder BA, Kemp SD. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* 1989; **246**: 1155-8.
59. Gill VS, Lima VD, Zhang W et al. Improved virological outcomes in British Columbia concomitant with decreasing incidence of HIV type 1 drug resistance detection. *Clin Infect Dis* 2010; **50**: 98-105.
60. Costagliola D, Lodwick R, Ledergerber B et al. Trends in virological and clinical outcomes in individuals with HIV-1 infection and virological failure of drugs from three antiretroviral drug classes: a cohort study. *Lancet Infect Dis* 2012; **12**: 119-27.
61. Margot NA, Lu B, Cheng A et al. Resistance development over 144 weeks in treatment-naive patients receiving tenofovir disoproxil fumarate or stavudine with lamivudine and efavirenz in Study 903. *HIV Med* 2006; **7**: 442-50.
62. Margot NA, Waters JM, Miller MD. In vitro human immunodeficiency virus type 1 resistance selections with combinations of tenofovir and emtricitabine or abacavir and lamivudine. *Antimicrob Agents Chemother* 2006; **50**: 4087-95.
63. Yerly S, Junier T, Gayet-Ageron A et al. The impact of transmission clusters on primary drug resistance in newly diagnosed HIV-1 infection. *AIDS* 2009; **23**: 1415-23.
64. Yerly S, Vora S, Rizzardi P et al. Acute HIV infection: impact on the spread of HIV and transmission of drug resistance. *AIDS* 2001; **15**: 2287-92.
65. Brenner BG, Roger M, Routy JP et al. High rates of forward transmission events after acute/early HIV-1 infection. *J Infect Dis* 2007; **195**: 951-9.
66. Wagner BG, Garcia-Lerma JG, Blower S. Factors limiting the transmission of HIV mutations conferring drug resistance: fitness costs and genetic bottlenecks. *Sci Rep* 2012; **2**: 320.
67. Society TEAC. European Guidelines for treatment of HIV infected adults in Europe. http://www.europeanaidsclinicalsociety.org/index.php?option=com_content&view=article&id=59:eacs-guidelines&catid=37:guidelines&Itemid=41 (28-11-2012 2012, date last accessed).
68. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. http://aidsinfo.nih.gov/contentfiles/lvguidelines/AdultandAdolescentGL.pdf (28-11-2012 2012, date last accessed).
69. Garcia-Lerma JG, Nidtha S, Blumoff K et al. Increased ability for selection of zidovudine resistance in a distinct class of wild-type HIV-1 from drug-naive persons. *Proc Natl Acad Sci U S A* 2001; **98**: 13907-12.

70. Violin M, Cozzi-Lepri A, Velleca R et al. Risk of failure in patients with 215 HIV-1 revertants starting their first thymidine analog-containing highly active antiretroviral therapy. *AIDS* 2004; **18**: 227-35.
71. Johnson VA, Calvez V, Gunthard HF et al. 2011 update of the drug resistance mutations in HIV-1. *Top Antivir Med* 2011; **19**: 156-64.
72. Li JZ, Paredes R, Ribaudo HJ et al. Low-frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: a systematic review and pooled analysis. *JAMA* 2011; **305**: 1327-35.
73. Palmer S, Kearney M, Maldarelli F et al. Multiple, linked human immunodeficiency virus type 1 drug resistance mutations in treatment-experienced patients are missed by standard genotype analysis. *J Clin Microbiol* 2005; **43**: 406-13.
74. Hance AJ, Lemiale V, Izopet J et al. Changes in human immunodeficiency virus type 1 populations after treatment interruption in patients failing antiretroviral therapy. *J Virol* 2001; **75**: 6410-7.
75. Margulies M, Egholm M, Altman WE et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 2005; **437**: 376-80.
76. Nijhuis M, van Maarseveen NM, Lastere S et al. A novel substrate-based HIV-1 protease inhibitor drug resistance mechanism. *PLoS Med* 2007; **4**: e36.
77. Dam E, Quercia R, Glass B et al. Gag mutations strongly contribute to HIV-1 resistance to protease inhibitors in highly drug-experienced patients besides compensating for fitness loss. *PLoS Pathog* 2009; **5**: e1000345.
78. van Maarseveen NM, Andersson D, Lepsik M et al. Modulation of HIV-1 Gag NC/p1 cleavage efficiency affects protease inhibitor resistance and viral replicative capacity. *Retrovirology* 2012; **9**: 29.
79. Fun A, Wensing AM, Verheyen J et al. Human Immunodeficiency Virus gag and protease: partners in resistance. *Retrovirology* 2012; **9**: 63.
80. Delviks-Frankenberry KA, Nikolenko GN, Pathak VK. The "Connection" Between HIV Drug Resistance and RNase H. *Viruses* 2010; **2**: 1476-503.
81. Vandamme AM, Camacho RJ, Ceccherini-Silberstein F et al. European recommendations for the clinical use of HIV drug resistance testing: 2011 update. *AIDS Rev* 2011; **13**: 77-108.
82. Arts EJ, Hazuda DJ. HIV-1 Antiretroviral Drug Therapy. *Cold Spring Harb Perspect Med* 2012; **2**: a007161.
83. Peuchant O, Capdepont S, Ragnaud JM et al. Primary resistance to enfuvirtide (T20) in recently HIV-1 infected, antiretroviral-naive patients from the ANRS Aquitaine Cohort. *Antivir Ther* 2007; **12**: 559-62.
84. Young B, Fransen S, Greenberg KS et al. Transmission of integrase strand-transfer inhibitor multidrug-resistant HIV-1: case report and response to raltegravir-containing antiretroviral therapy. *Antivir Ther* 2011; **16**: 253-6.
85. Boyd SD, Maldarelli F, Sereti I et al. Transmitted raltegravir resistance in an HIV-1 CRF_AG-infected patient. *Antivir Ther* 2011; **16**: 257-61.
86. UNAIDS. How to get to zero: Faster. Smarter. Better. http://www.unaids.org/en/media/unaids/contentassets/documents/unaidspublication/2011/JC2216_WorldAIDSday_report_2011_en.pdf (August 21st, 2012.
87. Garcia-Lerma JG, Otten RA, Qari SH et al. Prevention of rectal SHIV transmission in macaques by daily or intermittent prophylaxis with emtricitabine and tenofovir. *PLoS Med* 2008; **5**: e28.
88. Parikh UM, Dobard C, Sharma S et al. Complete protection from repeated vaginal simian-human immunodeficiency virus exposures in macaques by a topical gel containing tenofovir alone or with emtricitabine. *J Virol* 2009; **83**: 10358-65.

89. Subbarao S, Otten RA, Ramos A et al. Chemoprophylaxis with tenofovir disoproxil fumarate provided partial protection against infection with simian human immunodeficiency virus in macaques given multiple virus challenges. *J Infect Dis* 2006; **194**: 904-11.
90. Cong ME, Youngpairoj AS, Zheng Q et al. Protection against rectal transmission of an emtricitabine-resistant simian/human immunodeficiency virus SHIV162p3M184V mutant by intermittent prophylaxis with Truvada. *J Virol* 2011; **85**: 7933-6.
91. Abdool Karim Q, Abdool Karim SS, Frohlich JA et al. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* 2010; **329**: 1168-74.
92. Grant RM, Lama JR, Anderson PL et al. Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. *N Engl J Med* 2010; **363**: 2587-99.
93. Van Damme L, Corneli A, Ahmed K et al. Preexposure prophylaxis for HIV infection among African women. *N Engl J Med* 2012; **367**: 411-22.
94. Thigpen MC, Kebaabetswe PM, Paxton LA et al. Antiretroviral preexposure prophylaxis for heterosexual HIV transmission in Botswana. *N Engl J Med* 2012; **367**: 423-34.
95. Baeten JM, Donnell D, Ndase P et al. Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N Engl J Med* 2012; **367**: 399-410.
96. NIAID NIoAaID. NIH modifies "VOICE" HIV prevention study in women. 2011.
97. Hurt CB, Eron JJ, Jr., Cohen MS. Pre-exposure prophylaxis and antiretroviral resistance: HIV prevention at a cost? *Clin Infect Dis* 2011; **53**: 1265-70.
98. Prada N, Davis B, Jean-Pierre P et al. Drug-susceptible HIV-1 infection despite intermittent fixed-dose combination tenofovir/emtricitabine as prophylaxis is associated with low-level viremia, delayed seroconversion, and an attenuated clinical course. *J Acquir Immune Defic Syndr* 2008; **49**: 117-22.
99. Supervie V, Garcia-Lerma JG, Heneine W et al. HIV, transmitted drug resistance, and the paradox of preexposure prophylaxis. *Proc Natl Acad Sci U S A* 2010; **107**: 12381-6.
100. Yoshinaga T, Kobayashi M, Seki T et al. Antiviral Characteristics Of S/GSK1265744, An HIV Integrase Inhibitor Dosed By Oral Or Long-acting Parenteral Injection. *52nd Interscience Conference on Antimicrobial Agents and Chemotherapy*. San Fransisco, CA, USA, 2012.
101. Quinn TC, Wawer MJ, Sewankambo N et al. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. *N Engl J Med* 2000; **342**: 921-9.
102. Donnell D, Baeten JM, Kiarie J et al. Heterosexual HIV-1 transmission after initiation of antiretroviral therapy: a prospective cohort analysis. *Lancet* 2010; **375**: 2092-8.
103. Cohen MS, Chen YQ, McCauley M et al. Prevention of HIV-1 infection with early antiretroviral therapy. *N Engl J Med* 2011; **365**: 493-505.
104. von Linstow ML, Rosenfeldt V, Lebech AM et al. Prevention of mother-to-child transmission of HIV in Denmark, 1994-2008. *HIV Med* 2010; **11**: 448-56.
105. Warszawski J, Tubiana R, Le Chenadec J et al. Mother-to-child HIV transmission despite antiretroviral therapy in the ANRS French Perinatal Cohort. *AIDS* 2008; **22**: 289-99.
106. Townsend CL, Cortina-Borja M, Peckham CS et al. Low rates of mother-to-child transmission of HIV following effective pregnancy interventions in the United Kingdom and Ireland, 2000-2006. *AIDS* 2008; **22**: 973-81.
107. Montaner JS, Hogg R, Wood E et al. The case for expanding access to highly active antiretroviral therapy to curb the growth of the HIV epidemic. *Lancet* 2006; **368**: 531-6.

108. Granich RM, Gilks CF, Dye C et al. Universal voluntary HIV testing with immediate antiretroviral therapy as a strategy for elimination of HIV transmission: a mathematical model. *Lancet* 2009; **373**: 48-57.
109. WHO. Influenza (seasonal). http://www.who.int/mediacentre/factsheets/fs211/en/index.html (October 11th, 2012.
110. Storms AD, Gubareva LV, Su S et al. Oseltamivir-resistant pandemic (H1N1) 2009 virus infections, United States, 2010-11. *Emerg Infect Dis* 2012; **18**: 308-11.
111. Meijer A, Jonges M, Abbink F et al. Oseltamivir-resistant pandemic A(H1N1) 2009 influenza viruses detected through enhanced surveillance in the Netherlands, 2009-2010. *Antiviral Res* 2011; **92**: 81-9.
112. Hurt AC, Hardie K, Wilson NJ et al. Community transmission of oseltamivir-resistant A(H1N1)pdm09 influenza. *N Engl J Med* 2011; **365**: 2541-2.
113. Seibert CW, Rahmat S, Krammer F et al. Efficient transmission of pandemic H1N1 influenza viruses with high-level oseltamivir resistance. *J Virol* 2012; **86**: 5386-9.
114. Bouvier NM, Rahmat S, Pica N. Enhanced mammalian transmissibility of seasonal influenza A/H1N1 viruses encoding an oseltamivir-resistant neuraminidase. *J Virol* 2012; **86**: 7268-79.
115. Smith JS, Robinson NJ. Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review. *J Infect Dis* 2002; **186 Suppl 1**: S3-28.
116. Stranska R, Schuurman R, Nienhuis E et al. Survey of acyclovir-resistant herpes simplex virus in the Netherlands: prevalence and characterization. *J Clin Virol* 2005; **32**: 7-18.
117. Pillay D. Emergence and control of resistance to antiviral drugs in resistance in herpes viruses, hepatitis B virus, and HIV. *Commun Dis Public Health* 1998; **1**: 5-13.
118. Sauerbrei A, Liermann K, Bohn K et al. Significance of amino acid substitutions in the thymidine kinase gene of herpes simplex virus type 1 for resistance. *Antiviral Res* 2012.
119. Jacobson JG, Ruffner KL, Kosz-Vnenchak M et al. Herpes simplex virus thymidine kinase and specific stages of latency in murine trigeminal ganglia. *J Virol* 1993; **67**: 6903-8.
120. McMahon MA, Siliciano JD, Lai J et al. The antiherpetic drug acyclovir inhibits HIV replication and selects the V75I reverse transcriptase multidrug resistance mutation. *J Biol Chem* 2008; **283**: 31289-93.
121. McMahon MA, Siliciano JD, Kohli RM et al. Sensitivity of V75I HIV-1 reverse transcriptase mutant selected in vitro by acyclovir to anti-HIV drugs. *AIDS* 2010; **24**: 319-23.
122. Castor D, Low A, Evering T et al. Transmitted drug resistance and phylogenetic relationships among acute and early HIV-1-infected individuals in New York City. *J Acquir Immune Defic Syndr* 2012; **61**: 1-8.
123. UNAIDS. UNAIDS Report on the Global AIDS Epidemic 2010. http://www.unaids.org/globalreport (10-05-2012 2012, date last accessed).
124. Barth RE, van der Loeff MF, Schuurman R et al. Virological follow-up of adult patients in antiretroviral treatment programmes in sub-Saharan Africa: a systematic review. *Lancet Infect Dis* 2010; **10**: 155-66.
125. Hamers RL, Wallis CL, Kityo C et al. HIV-1 drug resistance in antiretroviral-naive individuals in sub-Saharan Africa after rollout of antiretroviral therapy: a multicentre observational study. *Lancet Infect Dis* 2011; **11**: 750-9.
126. Hemelaar J. Implications of HIV diversity for the HIV-1 pandemic. *J Infect* 2012.
127. Pingen M, Wensing AM, Boucher CAB et al. Effect of frequently transmitted M46I/L mutations on the in vitro selection of drug resistance against lopinavir. *International Workshop on HIV&Hepatitis Virus Drug Resistance and Curative Strategies*. Sitges, Spain, 2012; A166.

Chapter 10

Dutch Summary -
Nederlandse samenvatting
voor niet-wetenschappers

Curriculum Vitae

List of Publications

PhD Portfolio

Acknowledgements - Dankwoord

Nederlandse samenvatting voor niet-wetenschappers

Wereldwijd zijn er ongeveer 34 miljoen mensen besmet het humaan immunodeficiëntie virus, kortweg HIV. Wanneer een HIV geïnfecteerd individu niet behandeld wordt, zal deze na ongeveer 5 tot 10 jaar "acquired immunodeficiency syndrome" ofwel AIDS ontwikkelen. Het immuun systeem is dan zodanig verzwakt dat het geen weerstand meer kan bieden tegen virussen, bacteriën en schimmels waarvan gezonde personen niet ziek zouden worden.

Inmiddels zijn er goede medicijnen die de replicatie van HIV kunnen remmen. Een geschikte combinatie van medicijnen kan de replicatie van HIV zelfs (bijna) volledig onderdrukken. Nucleoside reverse transcriptase remmers (NRTI) lijken op nucleosiden, de bouwstenen voor de vorming van viraal DNA, maar stoppen reverse transcriptase zodra de NRTIs worden ingebouwd. In eerstelijns therapie worden meestal twee van deze NRTIs gegeven in combinatie met een derde medicijn. Vaak is dit een non-nucleoside reverse transcriptase remmer (NNRTI) die het reverse transcriptase enzym remt, of een protease remmer (PI) die het virale eiwit protease dat andere virale eiwitten knipt remt. Helaas kan HIV zich zo aanpassen dat het minder gevoelig wordt voor medicijnen, dit doordat het virus resistentie mutaties selecteert.

Medicijnresistente HIV varianten kunnen ook worden overgedragen. Bij maar liefst 1 op 10 nieuw gediagnosticeerde patiënten wordt een HIV variant met resistentie mutaties gevonden. Als er bij het selecteren van antivirale therapie geen rekening mee wordt gehouden dat de patiënt is geïnfecteerd met een resistente HIV variant kan dit bijdragen aan therapie falen.

Tijdens therapie falen worden andere resistentie mutaties gevonden dan bij nieuw gediagnosticeerde patiënten. Dit verschil is het grootst bij de mutatie M184V/I in reverse transcriptase. Dit is namelijk de meest voorkomende mutatie gedurende therapie falen terwijl deze mutatie maar zelden wordt gevonden in nieuw gediagnosticeerde patiënten. Het is bekend dat de aanwezigheid van deze mutaties ervoor zorgen dat het virus minder goed kan repliceren. Het zou zo kunnen zijn dat virus varianten met een lage replicatie capaciteit minder efficiënt worden overgedragen. In **hoofdstuk 2** laten we inderdaad zien dat resistente HIV varianten met een lagere replicatie capaciteit minder goed worden overgedragen dan medicijngevoelig HIV en medicijnresistent HIV met een hoog vermogen tot replicatie.

Indien een resistente HIV variant over wordt gedragen naar een nieuwe gastheer, kan HIV zich aanpassen aan de nieuwe omgeving waar geen selectiedruk van medicijnen is. Om dit te onderzoeken hebben we een overzicht gemaakt van de

beschikbare literatuur, wat beschreven is in **hoofdstuk 3**. Dit liet zien dat er drie mogelijkheden zijn voor resistentie mutaties in overgedragen HIV varianten. Ten eerste kunnen resistentie mutaties verdwijnen, wat vooral gebeurt als de resistentie mutatie een grote negatieve invloed heeft op de replicatie van het virus. Een tweede mogelijkheid is het selecteren van zogenaamde atypische varianten, dat zijn aminozuren die geen resistentie of wild type aminozuur zijn. De derde mogelijkheid is het persisteren van resistentie mutaties, wat verassend vaak werd geobserveerd. Dit kan worden veroorzaakt doordat sommige resistentie mutaties maar een klein effect hebben op de replicatie van HIV. Ook kan HIV additionele mutaties selecteren die ervoor zorgen dat de lage replicatie capaciteit van resistente HIV varianten wordt gecompenseerd. Als een virus met zo'n combinatie van mutaties wordt overgedragen, zijn er meerdere stappen nodig voor reversie naar wild type virus. Als alle mogelijke stappen de virale replicatie capaciteit verlagen, kunnen resistentie mutaties niet reverteren. We noemen dat compensatoire fixatie.

De voorgaande studies volgden kleine aantallen patiënten en gebruikten verschillende methoden om evolutie van resistente HIV varianten te onderzoeken. Daarom hebben we in **hoofdstuk 4** op een systematische manier en in meer detail gekeken naar de evolutie van HIV resistentie in een grotere groep patiënten. Hoewel de meeste resistentie mutaties de replicatie capaciteit van HIV verlagen, werd er bijna geen reversie van resistentie mutaties waargenomen. We verwachten dat dit komt door compensatoire mutaties die de replicatie capaciteit van virussen met resistentie mutaties verhogen.

In **hoofdstuk 5** hebben we een groep patiënten die met een vergelijkbaar virus met meerdere resistentie mutaties zijn gediagnosticeerd in detail bestudeerd. Hieruit bleek dat deze HIV variant minimaal 7 jaar lang via zowel homo- als heteroseksueel contact werd overgedragen naar tenminste 8 nieuwe patiënten. Dit konden we verklaren doordat het virus goed kon repliceren. Gelukkig was deze HIV variant ondanks de aanwezigheid van meerdere resistentie mutaties toch gevoelig voor de huidige medicijnen.

Een andere manier om te onderzoeken of resistentie mutaties persisteren is door gebruik te maken van nieuwe technieken die veel gevoeliger dan voorheen resistentie mutaties kunnen detecteren. We hebben deze moderne techniek in **hoofdstuk 6** gebruikt om te kijken of er virale varianten met additionele resistentie mutaties aanwezig waren in patiënten waarbij standaard technieken slechts één resistentie mutatie vonden. De afwezigheid van zulke additionele resistentie mutaties in het merendeel van de onderzochte patiënten maakt het waarschijnlijk dat deze patiënten geïnfecteerd zijn met een circulerende HIV variant met stabiele resistentie mutaties.

Een belangrijke vraag is of die circulerende HIV varianten met een beperkt resistentie profiel van invloed zijn op de werkzaamheid van de huidig beschikbare medicijnen. In **hoofdstuk 7** hebben we laten zien dat de meest voorkomende resistentiemutatie bij nieuw gediagnosticeerde patiënten, M41L in reverse transcriptase, geen invloed heeft op de selectie van resistentie tegen moderne NRTIs *in vitro*. Ook reageerden patiënten met een M41L HIV variant in de meeste gevallen goed op therapie, net zoals patiënten die met een wild type virus gediagnosticeerd waren. Dit betekend dat de detectie van M41L geen reden hoeft te zijn voor een meer ingewikkelde combinatietherapie met meer tabletten, grotere kans op bijwerkingen en hogere kosten.

In **hoofdstuk 8** beschrijven we een patiënt die gediagnosticeerd werd met een wild type virus. Echter, na het starten van therapie bleek dat de patiënt ook was geïnfecteerd met een tweede, medicijnresistente, HIV variant (een zogenaamde superinfectie) waardoor de eerstelijns therapie faalde. Deze casus laat zien dat zowel patiënten als clinici moeten uitkijken voor de gevaren van superinfecties.

Dit proefschrift laat zien dat het circuleren van HIV varianten met stabiele, beperkte resistentie patronen een belangrijke bron van overgedragen HIV varianten met resistentie mutaties is. Daarnaast toonden we aan dat sommige van deze varianten geen invloed hebben op de werkzaamheid van moderne medicijnen.

Curriculum Vitae

Marieke Pingen was born on September 22nd, 1984 in Nijmegen. She finished her secondary school at the Elzendaal College in Boxmeer in 2003. In the same year, she started the study "Molecular Life Sciences" at Maastricht University, where she focused on infectious diseases. In 2006 she obtained her bachelor's degree and started the master program "Immunity and Infection" at Utrecht University. As part of this study she performed a six month internship with Department of Pediatric Immunology at the Wilhelmina Children's Hospital Utrecht, where she investigated HSP60-induced regulatory T cells under the supervision of dr. Yvonne Vercoulen and prof. dr. Berent Prakken. Her second, nine month internship was done with the Department of Infectious Diseases and Immunology with the Virology Division at the Faculty of Veterinary Medicine, where she studied coronavirus replication of under supervision of dr. Marne Hagemeijer and dr. Xander de Haan. After obtaining her master's degree in 2008 she started her PhD project, which was a collaboration between prof. dr. Charles Boucher at the Department of Virology at Erasmus Medical Center and dr. Annemarie Wensing and dr. Monique Nijhuis at the Department of Medical Microbiology at the University Medical Center in Utrecht. The results of her PhD project are described in this thesis.

Marieke Pingen werd geboren op 22 september 1984 te Nijmegen. Ze behaalde haar HAVO diploma in 2001 en VWO diploma in 2003. In 2003 begon zij de opleiding "Moleculaire levenswetenschappen" aan de Universiteit Maastricht, waar zij zich focuste op infectieziekten. In 2006 behaalde ze haar bachelor diploma en begon de master "Immuniteit en infectie" aan de Universiteit Utrecht. Tijdens deze studie liep zij zes maanden stage op de afdeling Pediatrische Immunologie in het Wilhelmina Kinderziekenhuis, waar ze onderzoek deed naar HSP60-geinduceerde regulatoire T cellen onder begeleiding van dr. Yvonne Vercoulen en prof. dr. Berent Prakken. Haar tweede stage van negen maanden deed ze bij de afdeling Virologie, Departement Infectieziekten en Immunologie van de Faculteit Diergeneeskunde. Hier onderzocht ze replicatie van coronavirus onder begeleiding van dr. Marne Hagemeijer en dr. Xander de Haan. Na het behalen van haar master diploma in 2008 begon ze aan haar promotieonderzoek, een samenwerking tussen prof. dr. Charles Boucher van de afdeling Virologie van het Erasmus Medisch Centrum en dr. Annemarie Wensing en dr. Monique Nijhuis van de afdeling Medische Microbiologie van het Universitair Medisch Centrum. Het resultaat van haar onderzoek is beschreven in dit proefschrift.

List of Publications

Evolution and viral characteristics of a long-term circulating resistant HIV-1 strain in a cluster of treatment-naive patients. Hofstra LM, Nijhuis M, Pingen M, Mudrikova T, Riezebos-Brilman A, Simoons-Smit AM, Van Ham PM, Bierman WFW and Wensing AMJ. J Antimicrob Chemother, in press.

Deep sequencing does not reveal additional transmitted mutations in patients diagnosed with HIV-1 variants with single nucleoside reverse transcriptase inhibitor resistance mutations. Pingen M, van der Ende M, Wensing A, El Barzouhi A, Simen B, Schutten M, Boucher C. HIV Med. 2013 Mar;14(3):176-81.

Therapy failure resulting from superinfection by a drug-resistant HIV variant. Pingen M, Nouwen JL, Dinant S, Albert J, Mild M, Brodin J, Simen BB, Walsh S, Kayser M, van der Ende ME, Schutten M, Boucher CA. Antivir Ther. 2012;17(8):1621-5.

Application of cultured human regulatory T cells requires preclinical in vivo evaluation. Vercoulen Y, Guichelaar T, Meerding J, Emmelot M, Pingen M, Storm G, Coffer P, Sawitzki B, Martens A, Mutis T, Prakken B.J Allergy Clin Immunol. 2012 Mar;129(3):852-855.e3.

Telbivudine exerts no antiviral activity against HIV-1 in vitro and in humans. van Maarseveen NM, Wensing AM, de Jong D, Beilhartz GL, Obikhod A, Tao S, Pingen M, Arends JE, Hoepelman AI, Schinazi RF, Götte M, Nijhuis M. Antivir Ther. 2011;16(7):1123-30.

Evolutionary pathways of transmitted drug-resistant HIV-1. Pingen M, Nijhuis M, de Bruijn JA, Boucher CA, Wensing AM. J Antimicrob Chemother. 2011 Jul;66(7):1467-80

PhD Portfolio

Name	Marieke Pingen
Department	Department of Virology
Research School	Post-graduate School Molecular Medicine
PhD period	September 2008 – December 2013 (defence: May 2013)
Promotor	Prof. Dr. C.A.B. Boucher
Copromotors	Dr. A.M.J. Wensing & Dr. M. Nijhuis (UMC Utrecht)

Courses and meetings

2006	Radiation safety 5B (by TU Delft)
2008	3 weeks semi-intensive English course (by EAC Edinburgh)
2009	Get out of your lab-days (by MolMed)
2009	Workshop on viral evolution and molecular phylogenetics (by KU Leuven)
2009	Applied Bioinformatics (by MolMed)
2011	Research Management (by MolMed)
2011	Biostatistical Methods I: basic principles (by NIHES)
2008-2011	HIV journal club UMC Utrecht
2009-2012	HIV journal club ErasmusMC
2008-2012	Viro-immuno meeting UMC Utrecht/UU/WKZ (monthly)
2008-2012	Internal presentations at the department of MMB - Virology Group UMC Utrecht (twice a month)
2008-2012	"EWI-meeting", internal presentations at the department of MMB (monthly)
2010-2012	Internal presentations at the Department of Virology, Erasmus MC
2009-2012	Koepel meeting (UMC MMB internal conference, 3x oral presentation)
2008-2012	Europe HIV Resistance (EHR)/European Society Antiviral Resistance (ESAR) meetings (3x oral presentation, 1x poster presentation)

Attended seminars and symposia

2008-2013	Dutch Annual Virology Symposium (DAVS)
2008-2009	Netherlands Conference on HIV pathogenesis, prevention and treatment (NCHIV) (1x poster presentation)
2011-2012	MolMed day (2x poster presentation)
2012	Training the innate immunity: immunological memory in innate host defence

(Inter)national conferences: Oral and poster presentations

2009	Poster: Unexpected high prevalence of transmitted drug resistance among newly diagnosed patients in the Dutch central region (7th European HIV Drug Resistance Workshop, Stockholm, Sweden)

2010	Oral: Single Transmitted Drug Resistance Mutations: Not Always an Indicator of Transmission of More Extensive Resistance Profiles (International HIV and hepatitis virus drug resistance workshop, Dubrovnik, Croatia)
2010	Poster: Therapy Failure Caused by a Superinfection with a Resistant HIV-1 Strain (5th International workshop on HIV transmission in Vienna, Austria)
2010	Oral: Single transmitted drug resistance mutations may not function as indicator of transmission of more extensive resistance profiles anymore (5th international workshop on HIV transmission in Vienna, Austria)
2011	Poster: Single transmitted drug resistance mutations may not function as indicator of transmission of more extensive resistance profiles anymore (4th NCHIV Amsterdam, the Netherlands)
2011	Poster: The frequently transmitted M41L mutation in RT does not affect the *in vitro* selection of resistance against Tenofovir and Emtricitabine (International HIV and hepatitis virus drug resistance workshop, Los Cabos, Mexico)
2011	Oral: The frequently transmitted M41L mutation in RT does not affect the *in vitro* selection of resistance against Tenofovir and Emtricitabine. (6th workshop on HIV transmission in Rome, Italy)
2012	Poster: Effect of frequently transmitted M46I/L mutations on the *in vitro* selection of drug resistance against Lopinavir (International Workshop on HIV&Hepatitis virus drug resistance and curative strategies, Sitges, Spain)
2012	Oral: Diminished transmission efficacy of replication deficient resistant HIV-1 variants in ex vivo transmission model (7th workshop on HIV transmission in Washington DC)

Teaching

2009-2010	Supervision internship student Life Sciences (5 months)
2009-2010	Supervision internship student master Biomedical Sciences (9 months)
2012	Supervision internship University of Applied Sciences (10 months)
2012	Guest lecture University of Applied Sciences Nijmegen (Dutch and English)

Dankwoord/Acknowledgements

En dan ineens, na jarenlang experimenteren, schrijven, ploeteren, leren, frustratiemanagement, lezen, vallen en opstaan is het tijd om mijn dankwoord te schrijven. Vanaf het moment dat ik voor het eerst het UMC binnen fladderde wist ik dat het een intense, leerzame tijd zou gaan worden. Op deze plek wil ik graag iedereen die daaraan heeft bijgedragen bedanken, en een aantal mensen in het bijzonder.

Charles Boucher, Annemarie Wensing, Monique Nijhuis Charles, je zit altijd vol ideeën, concepten en experimenten, hebt een uitzonderlijk scherpe gedachtegang en laat je niet afremmen door 'aardse zaken' zoals samples en labgerommel. Anne, met je eindeloze energie hou je het belang van de patiënt in het oog, en altijd met een vleugje 'Anne-factor'. Monique, je vertaalt je goede ideeën naar degelijk labwerk en samen aan artikelen werken is een feestje. Mede dankzij jullie persoonlijke begeleiding heb ik naast belangrijke wetenschappelijke vaardigheden ook op persoonlijk vlak ontzettend veel geleerd de afgelopen jaren. Jullie hebben alle drie bijzondere kwaliteiten, maar samen is er een bijna magische synergie die enorm inspirerend is. Bedankt voor alles. Ik hoop dat jullie gedeelde enthousiasme en optimisme nog vele ideeën en inzichten op gaat leveren in de toekomst.

Antoinet, Carola, en Jacqueline Het was vaak een uitdaging om drie begeleiders met ongelooflijk drukke agenda's allemaal op dezelfde tijd, dezelfde plaats te krijgen. Gelukkig konden jullie me altijd helpen met al mijn verzoeken, vragen en ook met de nodige gezelligheid en recepten uitwisselingen. Bedankt!

HIV groep Behalve experimenten en deur-irritaties deelden we ook heel veel gezelligheid. Jori, we waren vanaf het begin lab-, wandel/step- en ijs-eet-maatjes, met veel discussies, peptalks en als het nodig was een kritische noot. We vullen elkaar goed aan en ik hoop dat dat in de toekomst zo blijft. Sue, always there in times of lab crises and need of native proofreading, but especially after work with yoga, Indian food, wine and trips. I'm looking forward to the next concert! Petra, naast je praktische hulp heb ik onze lichtpauzes, chocolademomenten, je humor en scherpe observaties enorm gewaardeerd. Pauline en Dorien, harde kern van de HIV groep, naast alle praktische hulp ook bedankt voor de vele gezellige momenten in de flow kast, jullie luisterende oor en vrolijk geduw op de MMB-gangen. Ik vind het stom dat het is afgelopen!

Students Next to all the work you've performed, I've learned a lot from (supervising) you. Iselle, Prince, Leander, thanks for your effort and all the best for the future.

UMC Utrecht Daarnaast wil ik ook alle andere fijne, gezellige, hulpvaardige UMC collega's bedanken voor een leuke tijd. In het bijzonder wil ik graag Arthur,

Pascal, Samira en Tania bedanken voor alle hulp met samples verzamelen en isoleren, sequencen en realtime pcr-en.

ErasmusMC It wasn't always convenient to work at two universities, but it also meant twice as many great colleagues and many opportunities. I've always felt welcome at Erasmus and enjoyed working both at the 3rd and the 16th/17th floor thanks to everyone. I'm sorry I couldn't be at the research meetings more often due to labour-intense lab experiments and UMC-meetings. I'd especially like to thank Dineke, David, Brooke, Azzania, Erhard, Nancy, Ineke and Martin for fun, discussions and great (literature) meetings.

Roomies I think good roomies are essential for successful PhD completion, because they provide necessary advice, cookies and procrastination. András, Bas, Elena, Ellen, Jori, Giske, Neeltje, Mike, Rutger, Steven and Xinglin: thanks for all the conversations, headbanging, (Portuguese) wine, walks in and around the UMC, food, roomy-boat trip, tea-moments and support.

EHR/ESAR It has been an honour, privilege and inspiration to meet and collaborate with so many bright, friendly and enthusiastic people from all over Europe. I owe you all great thanks, especially those of you who participated in the PERSIST-study (chapter 4).

Karolinska Institute Jan Albert, Mattias Mild and Johanna Brodin, it's been a great pleasure to work with you, and to learn about sequence analysis via our conference calls. Thank you.

AMC Dear Theo Geijtenbeek and Ramin Sarrami Forooshani, thanks for welcoming me into the field of dendritic cells and your lab. I hope our collaboration will soon result in our first but not last joint paper.

Additional collaborators Birgitte Simen (Roche), Manfred Kayser and Susan Walsh (Forensic Molecular Biology, ErasmusMC), thanks for the fruitful collaboration resulting in chapter 6 and 8.

Berent, Yvonne, Peter en Marne Jullie optimisme, kritische wetenschappelijke houding, enthousiasme en bevlogen begeleiding tijdens mijn master heeft een belangrijke rol gespeeld in mijn ontwikkeling, nogmaals bedankt!

Inge, Erik en Marc Bijzonder bedankt voor de therapeutische kopjes thee, jullie luisterende oren en wijze woorden.

Paranimfen Caroline, vriendinnen vanaf bachelor-dag-1, consigliere, ook al gaan we waarschijnlijk niet meer 'dreamteamen', ik hoop dat we altijd samen

arretjescake blijven eten en lachspieren blijven trainen. Sue, I love our cocktail-currency and hope we'll have many more trips to go.
You're both very good friends despite or maybe because we're so different yet so alike. I'm very happy you'll be there with me during the defence, you rock!

Vrienden en familie Elise, Wendy, Janine, Neeltje, Josine, Barbara, Emiel, Ewoud, Lili, "I&I-ers", Chris en Annie, nichten, en alle anderen die mijn leven leuker gemaakt hebben de afgelopen jaren: bedankt allemaal voor de nodige etentjes, spelletjesavonden/nachten, feestjes, whisky's, concerten, films/filmquizen, festivals en tripjes zodat ik af en toe niet aan HIV dacht, en het begrip wanneer ik nergens anders tijd voor had.

Ouders en zus Lieve pap en mam, jullie hebben me geleerd dat alles kan zolang je niemand schaadt en altijd met een open blik naar de wereld te kijken. Lievelingszus, bedankt voor het party-plannen, rinkelen, en het zussen zijn.
Ik hou van jullie.

Bart Je weet precies wanneer ik moet doorwerken, of het juist tijd is voor een ontspannende binnen-picknick of wandeling. Je bent er altijd voor joepie-bubbels of troostende knuffels. Kortom, m'n allerbeste vriendje. Bedankt!

Marieke

"Dream on, dream until your dreams come true"
— Steven Tyler

Propositions

The replicative capacity of HIV can impact the efficacy of transmission (this thesis)

Persistence of transmitted drug resistance mutations causing a decrease in replicative capacity can be explained by compensatory fixation (this thesis)

Onward transmission is an important source fuelling the epidemic of transmitted drug resistant HIV variants (this thesis)

Phylogenetic clustering of HIV can provide useful clinical and public health insights (this thesis)

The frequently transmitted HIV M41L variant does not impact the resistance pattern selected by tenofovir and therefore has limited clinical significance (this thesis)

"There are more proviruses in us than there is us in us"
— John Coffin

There is a significant difference between statistical significance and biological significance

When it comes to DNA, size does matter

"It is all right to make mistakes; nothing is perfect because with perfection, we would not exist"
— Stephen Hawking

"Life, in short just wants to be"
— Bill Bryson

"A little nonsense now and then, is cherished by the wisest men"
— Roald Dahl

Transmission and Evolution of Drug Resistant HIV-1 Variants

In at least 10% of newly diagnosed patients, HIV-1 variants harbouring resistance mutations in protease and reverse transcripase are detected. This thesis describes the impact of such resistance mutations on the transmission efficacy of HIV, the evolution of drug resistant HIV variants after transmission and the clinical impact of transmitted drug resistant viruses.

Marieke Pingen